PYRUVATE CARBOXYLASE:
AN IMMUNOLOGICAL STUDY

By

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A thesis presented for the degree of
Doctor of Philosophy.
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This thesis was written in the memory of Garry.

May biochemistry reveal his inner secret.

ERRATA

Page 1 Line 12 should read 'and more recently by Attwood and Keech (1984), Keech and Attwood (1985) and ....'

Page 11 Line 4 should be McLure *et al*., 1971b

Page 15 Line 9 from bottom should be McLure *et al*., 1971a

Page 25 Last line: replace the word 'of' with 'the'

Page 93 Line 5 from bottom: insert the word 'suggesting' after 'was present'.

Page 117 Last word 'was' should be omitted

Page 119 Line 6 should read Rohde *et al*., 1986

Bibliography should include:

Attwood, P.V., Wallace, J.C. and Keech, D.B. (1984) *Biochem. J.* 219, 243.

Phillips, D.G. (1966) *Sci. Amer.* (5) 215, 79.

It should be noted that:

Avidin is an inhibitor of pyruvate carboxylase activity.

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SUMMARY

1. Two sets of monoclonal antibodies directed against sheep liver pyruvate carboxylase (SLPC) have been produced. The first set (A) was screened using an enzyme-linked immunosorbent assay (ELISA). Twelve monoclonal cell lines were isolated. The subclass of each antibody was determined. None of these antibodies were able to inhibit the activity of SLPC. The second set (B) of monoclonal antibodies was screened by ELISA (using SLPC and other biotin carboxylases) and inhibition of enzyme activity assays. Antibodies 6, 12, 18, 42, 60, 78, 80, 98 and 113 from set B have been studied in more detail.

2. Antibodies 6, 12, 18, 42 and 113 were able to inhibit the acetyl-CoA dependent activity of SLPC. Antibodies 12 and 42 can also inhibit chicken liver pyruvate carboxylase (CLPC) activity. None of the inhibitory antibodies were shown to be directed at the biotin moiety.

   The inhibition of SLPC by antibodies 12 and 42, was shown to be reversed in the presence of oxaloacetate, suggesting that these antibodies may bind close to the second subsite within the enzyme's active site. However, $MgATP^{2-}$ also reversed the inhibitory effects of antibody 42 which would otherwise indicate antibody binding close to the first subsite. The inhibition by antibody 6 was increased in the presence of oxaloacetate again suggesting binding close to the second subsite. The other inhibitory antibodies were hardly affected by the presence of substrates or products of the enzyme.

3. Using avidin (which binds biotin tightly) in conjunction with electron microscopy, Johannssen et al. (1983) have determined the position of avidin binding on pyruvate carboxylase and thus the biotin prosthetic group and active site of the enzyme. Knowing the position of the biotin moiety, the dimensions of avidin and an anti-biotin IgG molecule, the area on the enzyme occluded by the binding of the latter two molecules was determined.

   The binding of avidin to pyruvate carboxylase reduced the binding of monoclonal antibodies 12 and 42 to the enzyme. Thus these antibodies bind within the area excluded by avidin. The binding of anti-biotin antibodies was shown to decrease the binding of antibodies 12, 42 and 80. Antibody 80 must bind outside the area excluded by avidin but within the anti-biotin exclusion area. The binding of the other inhibitory antibodies 6, 18 and 113 and the non-inhibitory antibody 60, was not affected by avidin nor anti-biotin antibodies implying that these antibodies do not bind within the avidin or anti-biotin exclusion areas.

4. Two lysyl residues on SLPC are known to be modified with trinitrobenzenesulphonate (TNBS) resulting in the loss of acetyl-CoA dependent activity. If acetyl-CoA is present during modification, one of these lysine residues is protected and there is no effect on catalytic activity (Ashman et al., 1973). Using TNBS modified SLPC, the position of these lysine residues was investigated by observing the binding of anti-TNP antibodies in the presence of avidin, anti-biotin and monoclonal antibodies. The most striking effect observed was the decrease in binding of antibody 80 in the presence

of TNP antibodies to SLPC modified both in the presence and absence of acetyl-CoA. This suggested that antibody 80 binds close to the position of the lysine which is not protected by acetyl-CoA during TNBS modification (Lys-B). In addition, a small significant decrease in the binding of TNP antibodies to SLPC in the presence of biotin antibodies was observed, suggesting that these antibodies bind close to the anti-biotin exclusion area.

5. The biochemical characterisation of the monoclonal antibodies was supported by electron microscopic studies which involved the physical location of the antibody binding sites on SLPC.

The inhibitory antibodies 6, 12, 42 and 113 were found to bind close to the intersubunit junction of the enzyme where the biotin prosthetic group is situated. For antibodies 12 and 42, this observation agrees with the previous biochemical evidence. The binding of antibodies 6 and 113 however, was not affected by the binding of avidin nor anti-biotin antibodies. Under the electron microscope, antibodies 6 and 113 did not lie completely over the intersubunit junction as did antibodies 12 and 42 and were therefore assigned to the side of the enzyme subunit, close to the intersubunit junction.

The inhibitory antibody 18, the binding of which is not affected by avidin, TNP antibodies or anti-biotin antibodies, was shown, by electron microscopy, to bind to the ends of the enzyme subunit, i.e. distal to the intersubunit junction. The method by which this antibody inhibits the activity of the enzyme needs to be investigated further.

The non-inhibitory antibody 60 was shown to bind to the ends of the enzyme subunit. Antibody 80 was shown to bind to the mid-section of the subunit. This is supported by the biochemical evidence which shows that antibody 80 binds within the anti-biotin antibody's exclusion area but not the avidin exclusion area. It follows that Lys-B is found in this region as well.

Based on the biochemical and electron microscopic experiments an epitope map of the binding positions of the monoclonal antibodies (Set B) was constructed.

6. The position of binding of anti-biotin IgG Fab fragments on SLPC was determined by electron microscopy and is in agreement with that determined by Johannssen et al. (1983) using avidin.

7. The cross reaction of Set B monoclonal antibodies with pyruvate carboxylase from sheep and chicken liver and yeast (YPC), transcarboxylase (TC) from P. shermanii and propionyl-CoA carboxylase from sheep liver (SLPCC) was investigated by ELISA. Antibody 60 was shown to bind to all of the biotin enzymes tested. Antibodies 12 and 42 bound to all enzymes except SLPCC. Antibodies 18, 80 and 113 bound weakly to CLPC and YPC only, while antibody 6 was specific for SLPC only. The observation that antibodies 12, 42 and 60 are able to recognise biotin carboxylases apart from the pyruvate carboxylases, supports the idea that there is some structural similarity amongst these enzymes and it is possible that they may have some evolutionary links.

STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge and belief, this thesis contains no material that has been previously published, or written by another person except where due reference is made in the text.

ACKNOWLEDGEMENTS

I wish to thank Professor W.H. Elliott for permission to undertake research in the Department of Biochemistry, University of Adelaide and to acknowledge the financial support of a Commonwealth Postgraduate Research Award for the duration of the project.

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To Dr Greg Goodall, Dr Yee-Sim Khew-Goodall, Dr Ian Cassady, Mr Filip Lim, Mr David Mottershead, Mr Mark Snoswell, Ms Susan Gayle and Dr Steven Gadd, many thanks for your advice, moral support and friendship during the course of this work. I am also grateful to Ms Jenny Brazier for her skilled technical assistance.

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Finally, I would like to thank Simon for his love and patience during the writing of this thesis.

ABBREVIATIONS

In addition to those accepted for use in the Journal of Biological Chemistry, the following abbreviations are used in the thesis:-

ACC	acetyl-CoA carboxylase
bp	base pairs
BSA	bovine serum albumin
CLPC	chicken liver pyruvate carboxylase
CoA	Coenzyme A
DTE	dithioerythritol
DTSP	dithio-bis-(succinimidyl propionate)
E-biotin	biotin enzyme
ENZ-biotin	biotin enzyme
ELISA	enzyme linked immunosorbent assay
Fab	monovalent fragment produced by papain cleavage of immunoglobulin molecule
FCS	foetal calf serum
HAT	hypoxanthine, aminopterin, thymidine
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
HT	hypoxanthine, thymidine
HPLC	high performance liquid chromatography
kDa	kilodaltons
NEM	N-ethyl morpholine
PBS	phosphate buffered saline
PC	pyruvate carboxylase
PCC	propionyl-CoA carboxylase
RLPC	rat liver pyruvate carboxylase
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SLPC	sheep liver pyruvate carboxylase
SLPCC	sheep liver propionyl-CoA carboxylase
TC	transcarboxylase
TNBS	2, 4, 6 - trinitrobenzenesulphonate
TNP	trinitrophenylated (e.g. TNP-enzyme)
s.a.	specific activity
YPC	yeast pyruvate carboxylase

CHAPTER 1

INTRODUCTION

1.1 PYRUVATE CARBOXYLASE - A BIOTIN DEPENDENT ENZYME

When studying the enzymes involved in the gluconeogenic pathway, and in particular the dicarboxylic acid shuffle, it was realised that an unknown enzyme must be involved in the conversion of pyruvate to phosphoenolpyruvate (or phosphopyruvate as it was then known) in chicken liver mitochondria. Subsequently a CO_2-fixing enzyme in chicken liver mitochondria was discovered which became known as pyruvate carboxylase (E.C. 6.4.1.1) (Utter and Keech, 1960). This discovery has prompted many investigations into the chemical, kinetic and physical properties of pyruvate carboxylase from bacterial to vertebrate sources. Most of this work has been reviewed by Scrutton and Young (1972), Utter et al. (1975) and more recently by Attwood and Keech (1984 and 1985) and Wallace and Easterbrook-Smith (1985). In addition, general reviews on biotin enzymes by Moss and Lane (1971) and Wood and Barden (1977) are available. The discussion following will therefore highlight areas of the field which are relevant to the work covered in this thesis.

Although pyruvate carboxylase was first described as an essential enzyme of the gluconeogenic pathway, it also plays an anaplerotic role in many non-gluconeogenic tissues in the regeneration of C_4 compounds of the tricarboxylic acid cycle. Pyruvate carboxylase belongs to a "family" of proteins known as the biotin dependent enzymes. All the well-characterised biotin carboxylases catalyse an analogous two step reaction which involves the ATP driven carboxylation of a biotin prosthetic group on the enzyme followed by the transfer of the carboxyl group to an acceptor molecule.

$$\text{E-biotin} + \text{ATP} + HCO_3^- \rightleftharpoons \text{E-biotin-}CO_2^- + \text{ADP} + P_i \quad (1.1)$$

$$\text{E-biotin-}CO_2^- + \text{acceptor} \rightleftharpoons \text{ADP} + P_i + \text{acceptor-}CO_2^- \quad (1.2)$$

Hence the overall reaction is:

$$\text{ATP} + HCO_3^- + \text{acceptor} \rightleftharpoons \text{ADP} + P_i + \text{acceptor-}CO_2^- \quad (1.3)$$

Table 1.1 shows the acceptor molecules and their carboxylated form for some biotin carboxylases.

1.2 SPATIAL SEPARATION OF SUBSITES ON BIOTIN ENZYMES

The active site of these enzymes is considered to be composed of two spatially separated subsites where biotin carboxylation occurs at one site (equation 1.1) and the transfer of the carboxyl group to the acceptor molecule occurs at the other (equation 1.2). The carboxyl carrier, a covalently attached biotin prosthetic group, has been proposed to oscillate between the two subsites on the enzyme. In pyruvate carboxylase from vertebrate liver, biotin is attached via its valeric acid chain in amide linkage through a specific lysine residue, 35 residues from the C-terminal end of the enzyme. The active site of pyruvate carboxylase depicting the spatial separation of the subsites is shown schematically in Figure 1.1.

Evidence for the separation of the two partial reactions of the biotin enzymes comes from many sources. Firstly, it has been shown by Alberts and Vagelos (1972) and Wood (1972) that the acetyl-CoA carboxylase from *E.coli* and transcarboxylase from *P.shermanii* are composed of three different protein components each with a different function. The carboxylation of the biotin is carried out by the biotin

carboxylase component while the transfer of the carboxyl group to the acceptor molecule is catalysed by the carboxyl transferase component. The biotin prosthetic group is contained on a small carboxyl carrier component. The three protein components of both transcarboxylase and acetyl-CoA carboxylase have been isolated and the partial enzyme reactions have been demonstrated in the isolated components (Wood, 1972; Lane and Polakis, 1975). Reconstitution of the three components to form the active transcarboxylase enzyme has also been achieved by Wood and his colleagues.

The 3-methylcrotonyl-CoA carboxylase from Achromobacter IVS and propionyl-CoA carboxylase from Mycobacterium smegmatis have been shown to consist of two types of polypeptide where the larger one contains the biotin prosthetic group as well as the biotin carboxylase function. Addition of the smaller polypeptide to the biotin containing chain restores overall enzymic activity (Schiele, 1975; Haase et al., 1984).

Pyruvate carboxylase isolated from the liver of several species of vertebrate is composed of four apparently identical polypeptides where each peptide contains all of the enzymatic functions required to carboxylate oxaloacetate. Product inhibition studies of pyruvate carboxylase from chicken and sheep have indicated that the biotin carboxylation and the transcarboxylation subsites on the enzyme are spatially separated (Barden et al., 1972; Ashman and Keech, 1975). This evidence is supported by ESR studies on the enzyme where the ESR spectrum of the bound manganese (II) is only affected by pyruvate and inhibitors of the transcarboxylation reaction, but not by acetyl-CoA or substrates of the biotin carboxylase reaction (Fung et al., 1976). Recent electron microscopic examinations of sheep, chicken and rat pyruvate carboxylase mounted for electron microscopy in the absence of acetyl-CoA, have shown the existence of a cleft along the longitudinal midline of each subunit

(Mayer et al., 1980). From this observation it was suggested that each subunit may be divided into two distinct domains. Hence it appears that the two partial reactions of the biotin enzymes studied to date, occur on spatially separated subsites whether as a consequence of being on separate subunits as for example in the case of transcarboxylase or on the same subunit as in the eukaryotic pyruvate carboxylases.

It is also of interest that for acetyl-CoA carboxylase from E.coli, transcarboxylase from P. shermanii, human pyruvate carboxylase and human propionyl-CoA carboxylase that the biotin prosthetic group is attached 35 residues from the carboxyl terminal end of the polypeptide (Sutton et al., 1977; Maloy et al., 1979; Freytag and Collier, 1984; Lamhonwah et al., 1987).

1.3 EVOLUTION OF BIOTIN DEPENDENT ENZYMES

1.3.1 Hypothesis for Common Ancestral Genes

On the basis of the similarities in their reaction mechanism, Lynen (1975) proposed that the biotin enzymes as they exist today may represent various stages in the evolution of the enzyme system from common ancestral genes. He proposed that at the lower end of the evolutionary scale, the biotin carboxylases are composed of unifunctional polypeptides where each polypeptide contributes a different but essential part of the active enzyme. For example, the biotin carboxylase, the transcarboxylase and the biotin containing components are each found on different polypeptides. This type of organisation is displayed by acetyl-CoA carboxylase from E.coli and Achromobacter and transcarboxylase from P.shermanii. The second stage in the evolution of the biotin enzymes was proposed to be the fusion of the genes which encoded the biotin

carboxylase function and the biotin carrier protein to form a bifunctional subunit. The carboxyl transferase activity would remain on a separate polypeptide. Initially it was thought that 3-methylcrotonyl-CoA carboxylase from Achromobacter and pyruvate carboxylase from P.citronellolis would fit this description. However, Goss et al. (1981) have presented data on the P.citronellolis enzyme which shows that antibodies against the α-subunit of pyruvate carboxylase, which was thought to contain only the biotin and the biotin carboxylase function, are able to inhibit the ATP/P_i exchange, the pyruvate/oxaloacetate exchange and overall activity. Antibodies against the β-subunit had no effect on any of these activities. This suggests that both of the subsites of the active centre of pyruvate carboxylase are found on the α-subunit. Pyruvate carboxylase and acetyl-CoA carboxylase from yeast are composed of four apparently identical, trifunctional polypeptides. That is, each polypeptide contains the biotin carboxylase, transcarboxylase and the biotin carrier components. In addition, yeast pyruvate carboxylase is allosterically activated by a range of acyl-CoA compounds and inhibited by L-aspartate.The pyruvate carboxylases from vertebrates such as sheep and chicken are composed of trifunctional polypeptides as well but also contain sites for the binding of the regulatory molecules, acetyl-CoA, α-ketoglutarate and glutamate. Lynen proposed that this transition of enzymes consisting of unifunctional polypeptides through to bi- and trifunctional chains to yield multifunctional enzymes may be a result of fusion and mutation of the genes which code for the polypeptides of the active site. Figure 1.2 depicts these different classes of the biotin dependent enzymes. It is of interest that no organism has been found that has different biotin enzymes which share common subunits. For example, both P.citronellolis and a thermophilic Bacillus contain two different biotin containing

peptides from pyruvate carboxylase and acetyl-CoA carboxylase (Fall et al., 1975 and Buckley et al., 1969). In addition, Fall and Hector (1977) showed that 3-methylcrotonyl-CoA carboxylase and geranyl-CoA carboxylase from P. citronellolis do not share common subunits.

1.3.2 Determination of Primary Structure of Pyruvate Carboxylase

If the biotin enzymes have evolved from common ancestral genes then it may be expected that some structural homology would be exhibited amongst the biotin enzymes provided that only limited divergence has occurred. Obviously, the combination of the crystal structures and the complete amino acid sequence of the biotin enzymes would be invaluable for determination of structural homology. Unfortunately, crystals of sufficient quantity and quality have not been isolated for any biotin enzyme. In the case of pyruvate carboxylase the large size of the subunit (110-120 kDa) has meant that until recently only a small amount of amino acid sequence has been determined. Since the N-terminal residue of most pyruvate carboxylases appears to be modified and unavailable for Edman degradation (Wallace and Easterbrook-Smith, 1985), the amino acid sequencing initially focussed on peptides which were associated with an important functional site. However, with sophisticated HPLC equipment available to separate peptide digests and automated gas phase sequencers able to sequence as little as 5-10 pmoles, more amino acid sequence is now being generated.

An increasing amount of work has been undertaken in the recombinant DNA area using cDNA probes synthesised on the basis of the amino acid sequence data from pyruvate carboxylase. Freytag and Collier (1984) used the amino acid sequence at the biotin attachment site for sheep liver pyruvate carboxylase (Rylatt et al., 1977) to synthesise a degenerate

oligonucleotide probe complementary to the codons for Met-Lys-Met-Glu-Thr. The probe was used to screen a human liver cDNA library. Three pyruvate carboxylase clones were obtained, from which a sequence of 96 amino acids of human liver pyruvate carboxylase has been generated. Using the insert of a human cDNA clone as a probe, Cassady et al. (1987a) have isolated rat liver pyruvate carboxylase (RLPC) clones from a rat liver cDNA library. 523 bp of coding sequence have been generated. The insert from a RLPC cDNA clone as well as an oligomer directed at the biotin attachment site were used as probes to isolate RLPC genomic clones. Using similar strategies, Morris et al. (1987) have inferred a large portion of the amino acid sequence of pyruvate carboxylase from the yeast Saccharomyces cerevisiae. It is hoped that this work will generate the complete DNA sequence and thus the amino acid sequence of chicken, rat, human and yeast pyruvate carboxylases.

1.3.3. Structural Homology Amongst the Biotin Enzymes

The sequence of amino acids surrounding the attachment site for the biotin prosthetic group has been determined for a variety of biotin enzymes (see Table 1.2.). Clearly the amino acid residues immediately adjacent to the lysine residue to which the biotin prosthetic group is attached, exhibit a high degree of sequence conservation for all of the biotin enzymes shown in the Table. The evidence for the homology adjacent to the biotin attachment site is consistent with the work of McAllister and Coon (1966), who showed that the holoenzyme synthetases (which attach biotin to the ϵ-amino group of a lysine residue) from rabbit liver, yeast and P.shermanii were mutually cross reactive forming active enzyme with apo-propionyl-CoA carboxylase of rat liver, apo-3 methylcrotonyl-CoA carboxylase from Achromobacter and apo-transcarboxylase from

P.shermanii. Thus it appears that the holoenzyme synthetases which attach biotin to the apo-enzyme, require a specific microenvironment around the lysine, to which the biotin will be attached. Mutants of the gene encoding the 1.3S biotinyl subunit of transcarboxylase from P. shermanii have been used to investigate the regions of this subunit important for biotinylation. Wood et al. (1987) and Shenoy et al. (1987) showed that mutations of the methionine residues adjacent to the lysine residue to which the biotin prosthetic group will be attached, do not affect biotinylation. These residues were found to be important however, for the carboxyl transferase reaction of the enzyme. In addition, Murtif and Samols (1987) have demonstrated that the presence of the hydrophobic side chain of the residue penultimate to the carboxy terminal is critical for biotinylation. They have proposed that successful biotinylation is dependent on the correct conformation of the carboxy terminal region with respect to the lysine to be biotinylated and, in turn, is dependent on the hydrophobicity of this region.

Although there is considerable sequence homology amongst the biotin enzymes around the biotin attachment site, until very recently there was little amino acid or DNA sequence data suggesting extensive homology beyond the sequence shown in Table 1.2. Using genomic or cDNA clones, the complete or partial amino acid sequence of some biotin enzymes has been ascertained. These biotin enzymes are transcarboxylase from P. shermanii (Murtif et al., 1985; Thornton et al., 1987), pyruvate carboxylase from human (Freytag and Collier, 1984), rat (Dr Ian Cassady, personal communication) and yeast (Morris et al., 1987), propionyl-CoA carboxylase from human (Lamhonwah et al., 1986 and 1987) and rat (Kraus et al., 1986), acetyl-CoA carboxylase from chicken (Takai et al., 1987) oxaloacetate decarboxylase from Klebsiella (Dr E. Schwarz, personal

communication) and a biotin containing peptide from tomato (Hoffman et al., 1987). This information has been used to demonstrate that some biotin enzymes do exhibit a degree of sequence similarity. For example, where the carboxyl terminal sequence data is available, the biotinyl lysine is found 35 amino acids from the carboxy terminus on each protein. Yeast pyruvate carboxylase and chicken acetyl-CoA carboxylase are exceptions, however. In addition, the sequence pro-x-pro is found 25-30 amino acids on the N-terminal side of the biotinyl lysine, in all biotin enzymes for which this data is available. The biotinyl moiety is believed to be able to oscillate between the two active subsites on biotin enzymes in order to shuttle carboxyl groups. Since prolines have a tendency to bend amino acid chains, Samols and his co-workers (1988) have suggested that the sequence pro-x-pro may be a candidate for a flexibility point which allows the biotinyl moiety to function. Again, yeast pyruvate carboxylase and chicken acetyl-CoA carboxylase are exceptions.

Comparison of the CoA ester binding peptides (predicted from the DNA sequence) from the β-subunit of rat propionyl-CoA carboxylase and the 12S monomer of transcarboxylase from P. shermanii, shows 50% identity and 63% similarity (if conservative amino acid substitutions are included) over the N-terminal 400 amino acids. Hence there has been considerable retention of amino acid sequence over large evolutionary distances. Similarly, the N-terminal 300 amino acids of the 5S subunit of transcarboxylase are 55% identical and 70% similar (with conservative substitutions) with oxaloacetate decarboxylase from Klebsiella. Comparison of the N-terminal 150 amino acids of the 5S subunit of transcarboxylase and yeast pyruvate carboxylase reveals 43% identity and 63% similarity (Samols et al., 1988).

Using the sequence data from human pyruvate carboxylase, propionyl-CoA carboxylase, E.coli acetyl-CoA carboxylase, transcarboxylase from P. shermanii, rat and yeast pyruvate carboxylase, Cassady et al. (1987b) compared the secondary structure propensity, hydrophobicity and charge profile of these biotin enzymes. Using these techniques, they observed significant structural similarity between the biotin enzymes.

This evidence offers some support to Lynen's hypothesis (1975) that the biotin enzymes have evolved from common ancestral genes and are a family of proteins. The biotin enzymes have different metabolic roles but share their mechanism of action, the latter of which may account for the conservation of critical areas of these enzymes.

1.3.4 Immunology of the Biotin Enzymes

Until 1984 there was no immunological evidence which supported antigenic similarity amongst the biotin enzymes (See Table 1.3). These studies were performed with native enzyme. Recently, antibodies raised against denatured sheep liver pyruvate carboxylase and sheep liver propionyl-CoA carboxylase have been shown to cross react with several of the biotin enzymes using an enzyme-linked immunosorbent assay (ELISA) system (Mottershead et al., 1984). This data is summarised in Table 1.3.

1.4 FURTHER STUDIES ON THE STRUCTURE AND MECHANISM OF PYRUVATE CARBOXYLASE

1.4.1. Subunit Structure

It is well accepted that the active form of pyruvate carboxylase from mammalian and avian sources is predominantly a tetramer. Active octomers of chicken liver pyruvate carboxylase, active dimers and monomers of rat liver pyruvate carboxylase (Taylor et al., 1978) and active dimers of pig liver pyruvate carboxylase (Warren and Tipton, 1974) have also been reported. The subunit molecular mass of the mammalian and avian pyruvate carboxylase ranges from 112-130 kDa and each subunit appears to contain one biotin moiety. Manganese is preferentially bound to pyruvate carboxylase from avian liver and is also bound to the enzyme from sheep liver and kidney, rat liver, calf liver and to a lesser extent to pig liver pyruvate carboxylase (Scrutton et al., 1973; McClure et al., 1971; Bais, 1974; Warren and Tipton, 1974). All pyruvate carboxylases examined to date have a modified amino-terminal group unavailable for Edman degradation and hence it is not possible to determine by sequencing whether the subunits are identical or not. However, no evidence has been presented which shows that the subunits are different (Wallace and Easterbrook-Smith, 1985).

1.4.2 Electron Microscopy

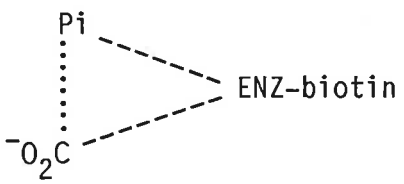
From electron microscopic examination, the three dimensional structure of pyruvate carboxylase from chicken liver was first reported to be a square planar tetramer with the identical subunits arranged at the corners of a square (Valentine et al., 1966). Conversely, a highly

purified preparation of the yeast enzyme appeared to be composed of rhombic structures (Valentine, 1968). In 1979 Goss and his co-workers and Cohen and her colleagues reported simultaneously that the square planar tetrameric molecule seen in earlier electron micrographs of the vertebrate pyruvate carboxylases was in fact a biotin-free contaminant. With pyruvate carboxylase from chicken, sheep and rat liver, they showed that the subunits of these enzymes were non-spherical and that they were arranged in a rhombic or splayed tetrahedral fashion where one pair of subunits was displaced above the plane of the second pair. Subsequently, a detailed three dimensional structure of chicken, sheep and rat liver pyruvate carboxylase was proposed by Mayer et al. in 1980. The tetramers of pyruvate carboxylase appeared to be tetrahedron-like structures consisting of two pairs of subunits in different planes orthogonal to each other with opposing pairs of subunits contacting on their convex surfaces. These workers showed that when pyruvate carboxylase was mounted for electron microscopy in the presence of acetyl-CoA, tetrahedral shaped molecules were observed. In the absence of acetyl-CoA splayed or seemingly "flattened" tetrahedra, dimers and monomers were evident. With the aid of 'tilting experiments', it was shown that acetyl-CoA helped to preserve the native configuration of the molecule and that without it, the tetrahedron seemed to be "flattened" by the mounting and staining process. In addition, when pyruvate carboxylase was mounted in the absence of acetyl-CoA, a cleft was often observed running down the longitudinal midline of each subunit in the tetramer suggesting a division of the subunit into two domains. The dimensions of each subunit were determined as being 8.5 nm in length by 7.0 - 7.5 nm in width by 3.5 - 3.75 nm in depth. Models of sheep liver pyruvate carboxylase (SLPC) mounted in the presence of acetyl-CoA are shown in Figure 1.3.

Using electron microscopy in conjunction with the protein avidin which is known to bind biotin very tightly ($K_d = 10^{-15}M$), the position of the biotin prosthetic group on pyruvate carboxylase was deduced by Johannssen et al. (1983). They proposed that the biotin prosthetic group was located on the external faces of each subunit of pyruvate carboxylase, probably within 3 nm of the intersubunit junction (for more details see section 4.2.2.2). Models and diagrammatic views of the complexes between avidin and chicken liver pyruvate carboxylase are shown in Figure 4.12.

1.4.3 The active Site of Pyruvate Carboxylase

The carboxylation of biotin takes place in the first subsite of pyruvate carboxylase (See equation 1.1). For this reaction to occur ATP, Mg^{2+}, HCO_3^-, acetyl-CoA and biotin attached to the enzyme must be present. The first partial reaction of pyruvate carboxylase does not occur without the cleavage of ATP. Many reaction mechanisms have been proposed which describe the role of ATP in biotin carboxylation (Kaziro et al., 1962; Scrutton and Utter, 1965; Polakis et al., 1972 and 1974). After incubation of pyruvate carboxylase with Mg^{2+}, ATP and HCO_3^-, Phillips et al. (1981) and Wallace et al. (1985) isolated an enzyme-bound carboxy-phosphate species putatively described below.



They showed that the carboxyl group was not transferred to pyruvate unless acetyl-CoA served to transfer the carboxyl group from the biotin carboxylation subsite on the enzyme to the biotin prosthetic group.

After biotin carboxylation at the first subsite, the carboxybiotin group moves to the transcarboxylation site on the enzyme. Pyruvate has been shown to be a signal for the carboxybiotin to move to the second subsite (Easterbrook-Smith et al., 1976). Goodall et al. (1981) showed that other substrates for pyruvate carboxylase as well as some inhibitor analogues can also trigger the carboxybiotin to move to the second subsite. Like pyruvate these compounds contain both oxo and carboxyl groups. Compounds which lacked either the oxo or carboxyl group were unable to evoke this movement. The pyruvate analogue 2-oxobutyrate results in a much reduced rate of carboxylation in comparison to pyruvate and is therefore measurable by conventional methods. Using 2-oxobutyrate it was postulated that the carboxybiotin existed in two forms or states in equilibrium with each other. In State I the carboxybiotin was bound to the first subsite whereas in State II the carboxybiotin was not bound but in the vicinity of the first subsite. When Mg^{2+} was present the enzyme bound form of the carboxybiotin complex (State I) existed in two forms where the Mg^{2+} was either bound or unbound. It was proposed that Mg^{2+} "holds" the carboxybiotin at the first subsite whereas α-oxo acids such as pyruvate induce the complex to move to the second subsite (Goodall et al., 1981). Using quenched flow rapid reaction techniques, Attwood et al. (1984) proposed that high concentrations of Mg^{2+} inhibited the overall enzyme activity by causing the transfer of the carboxyl group from biotin to pyruvate to become rate limiting. In addition, the effectiveness of the substrate as a signal for the carboxybiotin to leave the first subsite was related to its effect on the affinity of the enzyme-carboxybiotin complex for Mg^{2+}.

Several mechanisms have been proposed for the transfer of the carboxyl group from the carboxy-biotin to the acceptor substrate in the second subsite. These include a concerted mechanism (Mildvan et al.,

1966; Fung et al., 1976), a carbanion mechanism (Stubbe and Abeles, 1977; Stubbe et al., 1980) and an enolate anion relay mechanism (Goodall et al., 1983). The research involved in elucidating the carboxyl transferase mechanism has been reviewed by Keech and Attwood (1985), Wallace and Easterbrook-Smith (1985) and Attwood and Keech (1984).

1.4.4 Acetyl-CoA

Pyruvate carboxylase from different sources shows varying dependence on the presence of acetyl-CoA for activity. For example, the enzyme from chicken liver is completely dependent on acetyl-CoA, the enzyme from rat and sheep liver shows some acetyl-CoA independent activity under special assay conditions (Ashman et al., 1972; McClure et al., 1971 b) whereas the enzyme from P. citronellolis exhibits no dependence on acetyl-CoA at all (Taylor et al., 1972; Seubert and Remberger, 1961). Yeast pyruvate carboxylase is active in the absence of acetyl-CoA but activity is stimulated by acetyl-CoA. This stimulation is dependent on the concentration of K^+ and HCO_3^- in the assay (Myers et al., 1983). Both the biotin carboxylation and the transcarboxylation reactions are enhanced in the presence of acetyl-CoA (Ashman et al., 1972; Ashman et al., 1973; McClure et al., 1971; Scrutton and White, 1972).

It has been demonstrated using a flow dialysis technique that chicken liver pyruvate carboxylase (CLPC) has four binding sites for acetyl-CoA per tetramer (Frey and Utter, 1977).

The amino acids involved in the binding of acetyl-CoA to the enzyme are unknown. In 1968, Keech and Farrant demonstrated that a lysyl residue in sheep kidney pyruvate carboxylase was essential for acetyl-CoA dependent activity. This result was supported by Ashman et al. (1973) who showed that treatment of sheep pyruvate carboxylase with 2, 4,

6-trinitrobenzenesulphonic acid (TNBS) inactivated the acetyl-CoA dependent activity but activated the acetyl-CoA independent activity. Since the latter activity was still observed, this suggests that the modified lysine did not form part of the catalytic site but rather was essential for the regulatory function of acetyl-CoA. It is uncertain whether the TNBS modified lysine forms part of the acetyl-CoA binding site or whether the lysine is distant from this site. Desensitisation to acetyl-CoA by modification with TNBS has been reported for other pyruvate carboxylases (Scrutton and White, 1973; Libor et al., 1978; Osmani et al., 1981).

A second function of acetyl-CoA appears to be one of stabilisation of the enzyme's structure. All vertebrate pyruvate carboxylases lose enzymic activity below 3-4units/ml in the absence of acetyl-CoA (Ashman et al., 1972). HPLC and electron microscope studies have revealed that below 3 - 4 units/ml acetyl-CoA serves to preserve the tetrameric structure of the enzyme. If acetyl-CoA is added after dilution, further inactivation does not occur but there appears to be a reassociation of inactive monomers and dimers to form inactive tetramers. Once diluted, SLPC activity cannot be reactivated to its former activity but some reactivation has been observed with CLPC (Attwood and Wallace, unpublished results; Khew et al., 1983). It has been proposed that dilution of the enzyme to less than 4 units/ml results in both a conformational change in the enzyme yielding an inactive enzyme tetramer and dissociation of the tetramer into inactive dimers and monomers. The inactive tetramers have a greater diameter than active tetramers and appear to be flattened when viewed under the electron microscope.

It has been suggested that acetyl-CoA exerts its allosteric effect by binding to the enzyme and preventing a conformational change (Attwood and Keech, 1984). Conversely, the current view is that allosteric effectors

exert their effect by inducing a conformational change. To detect a conformational change in the enzyme upon the binding of acetyl-CoA, methods such as optical rotary disperson, protein fluorescence and reacting enzyme sedimentation have been employed with little success (Frey and Utter, 1977; Utter et al., 1975; Ashman et al., 1972; Scrutton and Utter, 1967). However, McGurk and Spivey (1979) demonstrated that the binding of acetyl-CoA to chicken pyruvate carboxylase resulted in a 10% quenching of a fluorescent probe suggesting that a conformational change had occurred. In addition, Frey and Utter (1977) observed small spectral changes in the UV-spectrum upon the binding of acetyl-CoA to chicken pyruvate carboxylase.

1.5 MONOCLONAL ANTIBODIES AND THEIR USES

In 1975 Köhler and Milstein reported the derivation of continuous cultures of specific antibody-producing cells through somatic cell hybridisation of mouse myeloma cells with lymphocytes from the spleens of immune mice. Each hybrid line produces an antibody directed against a single antigenic determinant - a monoclonal antibody.The tissue cultured cells may be frozen and recovered later or grown up in culture where they secrete 10-60 g/ml antibody into the culture fluid. The cells can also be injected into animals (mice) which form tumours. Large amounts of antibody may be recovered from tumour bearing animals' (as high as 1-10mg antibody/ml of body fluid) sera or ascites fluid.Thus a perpetual supply of an antibody of defined specificity is assured. Another advantage of hybridoma technology is the ability to produce a monoclonal antibody with impure antigens since cells producing antibodies against other impurities or determinants are discarded in the cloning procedure. Several

descriptions of the hybridoma technique have been published (Kennett et al., 1980; Kwan et al., 1980; McKearn et al., 1979; Herzenberg et al., 1978; Pearson et al., 1980; Galfré and Milstein, 1981; Oi and Herzenberg, 1980).

In contrast, antisera from conventionally immunised animals contain a heterologous mixture of antibodies with varying affinities, cross reactivities and effector functions. The antibodies may be directed towards different antigens in the immunising material, different determinants on a single antigen and different antibodies which are able to 'fit' a single determinant. To obtain a monospecific reagent, absorption of sera with unwanted antigens must be carried out.

The production of monoclonal antibodies in large amounts has had a broad impact in clinical medicine, scientific development and industry. Monoclonal antibodies have been produced against an enormous range of substances such as viruses, bacteria, parasites, cancer cells, blood group substances, transplantation antigens, hormones, proteins and enzymes. Some applications of monoclonal antibodies to neuroscience research, biology and medicine have been reviewed (Valentino et al., 1985; Yelton and Scharff, 1981; Pollock et al., 1984).

More specifically, monoclonal antibodies have been used extensively in research to study the structure and the relationship between structure and function of enzymes and proteins. Five examples of the use of monoclonal antibodies for this purpose follow:

1. The acetylcholine receptor

The acetylcholine receptor (AChR) is found at the tips of muscle membrane folds opposite nerve endings. Acetylcholine (ACh) is released in response to nerve impulses and moves across the synaptic cleft to its receptor.This results in the opening of the cation channel in the receptor which allows Na^+ into the muscle cell and then contraction of the muscle. Snake venom α-toxins bind to the receptor at or near the ACh binding site and block the receptor function (Karlin, 1980; Changeux, 1981; Tzartos and Changeux, 1983). The electric organ of the fish Torpedo is a rich source of AChR. The Torpedo receptor has a subunit stoichiometry of $\alpha_2\beta\gamma\delta$ (Rafferty et al., 1980; Changeux, 1981) with a molecular weight of 250,000. Many monoclonal antibodies have been produced against the AchR from fish electric organs and mammalian muscles.

The monoclonal antibodies have been used to show that there is structural similarity (at least at the antigenic determinants) amongst the different subunits of the receptor since they cross react with more than one subunit (Tzartos and Lindstrom, 1980). This has been confirmed by sequence analysis (both amino acid and DNA) of the subunits (Rafferty et al., 1980; Noda et al., 1983). The extensive sequence homology suggests that the receptor may have evolved through duplication and reduplication of a primordial subunit and that immunological cross reaction is a consequence of the resulting sequence homologies. Some monoclonal antibodies cross react between AchR from different species. The best cross reaction has been observed with antibodies which recognise the α-subunit (Tazartos and Lindstrom, 1980; Tzartos et al., 1981). At least three monoclonal

antibodies have been isolated which are thought to bind at or near the acetylcholine binding site since they are inhibited from binding to the receptor by α-toxin. Thus, these antibodies are additional probes to study the acetylcholine binding site (Mochly-Rosen and Fuchs,1981; James et al., 1980). Monoclonal antibodies have been used to affinity purify the receptor although affinity purification using the α-toxins has already been successful. Monoclonal antibodies have been isolated which inhibit channel opening in the Torpedo receptor. These antibodies are not directed at the toxin binding sites and bind to the α-subunit or the α- and β-subunits.

Epitope mapping studies and peptide analysis have revealed that most antibodies against AchR bind to a region on the α-subunit known as the MIR (main immunogenic region) (Tzartos and Lindstrom, 1980; Tzartos et al., 1981 and 1983; Swanson et al., 1983). This region is found on the extracellular part of the α-subunit but away from the α-toxin and ACh binding site (Tzartos and Lindstrom, 1980; Tzartos et al., 1981). Antibodies which bind to this region are very crossreactive across species suggesting that this region is conserved across species. The MIR does not contain the ion channel since antibodies to this region do not affect channel function.

Monoclonal antibodies to AchR have been used to study the human disease myasthenia gravis, the symptoms of which produce muscular fatigue and weakness. Myasthenia gravis is thought to be an autoimmune disease where antibodies against AChR are produced. The antibodies cause loss of AChR receptors by complement mediated lysis of receptor membranes and crosslinking of receptors, resulting in an increase in internalisation and in the rate of degradation of the

receptors. Rabbits injected with Electrophorus AChR produce antibodies against the receptor and show symptoms of human myasthenia gravis. Injection of a single monoclonal antibody to the receptor in rats, mice and guinea pigs has also induced the disease. Some monoclonal antibodies that bind to the extracellular surface of AChR do not induce myasthenia, however those that do cause the disease bind to the α-subunit (Tzartos and Lindstrom, 1980; Lindstrom and Engel, 1981; Drachman, 1981). In fact the majority of antibodies in sera from most patients with myasthenia gravis were directed against the MIR. In vitro studies with muscle cells in culture has shown that monoclonal antibodies directed at the MIR increase the internalisation and degradation rate of AChR.

From the work cited here, it is clear that monoclonal antibodies have been and will continue to be an invaluable tool in the elucidation of the structure and the relationship of structure to function of the receptor for acetylcholine.

2. The L7/L12 protein of the 50S ribosomal subunit

The protein designated L12 is contained within the E.coli 50S ribosomal protein. Its N-acetylated form L7 is also present and the two proteins are collectively known as L7/L12. Two dimers of L7/L12 are believed to exist in each 50S subunit. The protein is involved in GTP hydrolysis during protein synthesis and is therefore essential for function. To gain a wider understanding of the L7/L12 protein's function, the location of the four copies of L7/L12 on the ribosome was desired in order to identify structurally and functionally important domains on the 50S ribosomal protein.

Two monoclonal antibodies were prepared against L7/L12. They were shown to recognise different determinants on L7/L12. One was directed at the N-terminal while the other recognised a C-terminal epitope.This was determined by probing nitrocellulose blots of L7/L12 which had been proteolysed and electrophoresed. (The amino and carboxy terminal peptides were known). These antibodies were shown to prevent association of the elongation factor, EF-G, with the ribosome, to inhibit polyphenylalanine synthesis and ribosome dependent GTPase activity (Sommer et al., 1985). Using these antibodies in conjunction with immune electron microscopy, the position of both the L7/L12 dimers on the ribosome 50S subunit was determined (McKuskie Olsen et al., 1986). One dimer exists in a folded conformation on the subunit body and the second dimer is found in the subunit stalk in an extended conformation. This study brought an end to the conflicting reports for the placement of the two dimers on the ribosome.

3. Rat brain hexokinase

Hexokinase (ATP: D-hexose-6-phoshotransferase E.C. 2.7.1.1) catalyses the first step of the glycolytic pathway where glucose and $Mg.ATP^{2-}$ are converted to glucose 6-phosphate (G6-P) and ADP. Like the biotin carboxylases, hexokinase is a multifunctional enzyme. It must possess binding sites for its substrates, for the product and allosteric effector G6-P as well as a binding domain for the interaction with the outer mitochondrial membrane. Rat brain hexokinase is a single polypeptide chain of molecular weight, 98000. Limited proteolysis of this enzyme results in loss of binding capability but not catalytic

activity (Wilson, 1980; Rose and Warms, 1967; Kurokawa et al., 1982) indicating that the binding and catalytic domains are spatially separated on the enzyme.

To further investigate the relationship of structure to function of rat brain hexokinase, Finney et al. (1984) and Wilson and Smith (1985) have characterised seven monoclonal antibodies which bind to native rat brain hexokinase. They studied the effect of the antibodies on such functions as catalytic activity, binding to mitochondria, inhibition of enzyme activity by G6-P, release of hexokinase from the mitochondrial membrane in the presence of G6-P and the effect of substrates on the binding of the antibodies. By incubating all pairs of monoclonal antibodies with hexokinase it was determined whether the binding of the pairs were mutually exclusive, independent or overlapping. The area excluded by an antibody binding was assumed to be $35A^{o}$ in diameter (Tzartos et al., 1981). Using both one and two dimensional peptide mapping with electroblotting and immunoprobing techniques, the position of binding of each antibody along the primary structure of rat brain hexokinase was determined. In conjunction with the findings on the effects of the antibodies on the function of the enzyme and the epitope mapping studies, Wilson and Smith were able to construct a three-dimensional model of the enzyme. The model relates the immunological to the functional and structural aspects of the enzyme.

4. Lysozyme

Hen egg white lysozyme c (E.C. 3.2.1.17) is a small enzyme which cleaves the polysaccharide component of bacterial cell walls and is composed of a single polypeptide of 129 amino acids of molecular

weight 14600. The complete amino acid sequence is known and the three dimensional structure has been elucidated by X-ray crystallography (Phillips, 1966). Smith-Gill and her co-workers (1982, 1984 a,b) have located the epitopes on hen egg white lysozyme for a set of monoclonal antibodies directed against this enzyme. They have shown that antigenic regions defined by these antibodies in fact represent structural domains defined by the tertiary structure of the enzyme. More specifically, they have used these antibodies to study the molecular nature of antibody recognition of proteins to gain a better understanding of the mechanisms underlying antibody diversity.

5. Phenylanaline hydroxylase, Epoxide hydrolase

Monoclonal antibodies have also been used in the determination of differences or similarities in the antigenicity of the same protein from different sources. For example Choo *et al*. (1981) have characterised four monoclonal antibodies to the enzyme phenylanaline hydroxylase (E.C. 1.14.16.1) from monkey liver. Two of these antibodies also bind to phenylanaline hydroxylase from human, rat and mouse liver. The other antibodies bind only to the enzyme from human and monkey liver as determined by removal of enzyme activity in solution by antibody bound to Sepharose beads. In addition, Wolf *et al*. (1983) used thirteen monoclonal antibodies raised against rat liver epoxide hydrolase (E.C. 4.2.1.63) to determine antigenic similarities of epoxide hydrolase from rat, mouse, guinea pig, Syrian and Chinese hamster, rabbit, ape and human liver. They found that the monoclonal antibodies bound well to epoxide hydrolase from Syrian hamster and mouse. Only a few antibodies reacted with the guinea pig, ape or human liver enzyme. Two antibodies reacted well with

nearly all species tested suggesting that these antibodies may be directed at determinants which are conserved across species and thus could be critical functional sites on the enzyme.

The few examples cited here from the vast amount of literature that is now available on monoclonal antibodies, shows the use of these antibodies in answering a wide range of questions dealing with the structure of proteins and their function. Questions such as, 'Where do the antibodies bind on the protein?', have been answered by epitope mapping techniques combined with immunoprecipitation or electroblotting and immuno-probing of either proteolytic digests or denatured subunits of the protein. In addition immune electron microscopy has been useful for larger proteins. This information has been taken in conjunction with the effect of the antibody binding on the function of the protein, to build a picture of the relationship of structure to function. In addition, monoclonal antibodies have also been used to determine conservation of protein structure (at the antigenic determinants in question) across species, perhaps giving some clues as to the evolution of the protein in question.

1.6 Aims of the project

One of the aims of our research group is to study the evolutionary relationship amongst the group of enzymes known as the biotin carboxylases. As discussed in section 1.3 it has been proposed that the biotin carboxylases have evolved by fusion of the genes which code for the different functional domains of the biotin enzymes (Lynen, 1975).

Some members of our research group are actively involved in obtaining the DNA sequence encoding of different biotin enzymes. After this has

been achieved, comparisons may be made amongst the inferred protein sequences (including the sequences already available - see section 1.3.3) to determine any homologies and thus offer more support for the hypothesis of common ancestral genes. The other approach in studying the evolutionary relationship amongst the biotin enzymes, is an immunological one. This is based on the assumption that cross reaction of an antibody between two proteins indicates some structural similarity at that determinant. By observing the binding of different antibodies directed at many determinants on different biotin enzymes, a pattern of the structural similarity of the biotin enzymes may be ascertained.

To begin the immunological study, the present research project involved the preparation of a set of monoclonal antibodies against sheep liver pyruvate carboxylase and the characterisation of the binding of these antibodies to the sheep liver enzyme. This involved, in short, the determination of the effect of antibody binding on the function of the enzyme and location of a binding position for each antibody on the enzyme. Binding to other available biotin enzymes was also determined.

At the commencement of the project no immunological cross reaction amongst different biotin enzymes had been observed. (Cross reaction has since been observed by Mottershead and his co-workers (1984) using antibodies raised against SDS-denatured biotin enzymes). Amino acid residues on the surface of proteins are known to change more rapidly during protein evolution than internal residues (Schulz and Schirmer, 1979). It was thought that common determinants amongst the biotin enzymes may be composed of largely internal residues and hence be poorly immunogenic when the native enzyme was used as an immunogen. Hence the few antibodies that are raised against the common determinants are not readily detectable in a polyclonal serum. Using the monoclonal antibody technique, it was hoped that those clones producing antibodies against

common determinants could be isolated. In any case, the production of monoclonal antibodies against pyruvate carboxylase would provide invaluable tools for studying the structure/function relationships of the enzyme.

Table 1.1 The Principle Substrates and Products for some Biotin Enzymes

Biotin Carboxylase	E.C. No.	Substrate	Carboxylated Product
Pyruvate carboxylase	6.4.1.1	pyruvate	oxaloacetate
Acetyl-CoA carboxylase	6.4.1.2	acetyl-CoA	malonyl-CoA
Propionyl-CoA carboxylase	6.4.1.3	propionyl-CoA	methyl-malonyl-CoA
3-methylcrotonyl-CoA carboxylase	6.4.1.4	3-methylcrotonyl-CoA	3-methylglutaconyl-CoA
Transcarboxylase*	2.1.3.1	pyruvate	oxaloacetate

Biotin Decarboxylase	E.C. No.	Carboxylated Substrate	Decarboxylated Product
Oxaloacetate decarboxylase	4.1.1.3	oxaloacetate	pyruvate
Methyl-malonyl-CoA decarboxylase	4.1.1.41	methyl-malonyl-CoA	propionyl-CoA

* The carboxyl donor (methyl-malonyl-CoA) replaces ATP and HCO_3^- in the first partial reaction. Propionyl-CoA is a product of this reaction. The other biotin carboxylases utilise bicarbonate as the carboxyl donor.

Table 1.2 Comparison of the Amino Acid Sequences Adjacent to the Biotin Group in Biotin Carboxylases

a	Chicken PCC	Met	Gly	Gln	Glu	Ile	Tyr	Arg	-	-	-	-	BCT	Met	Gln	Asn	Ser	Met	Ile	Ala	Ala	Lys
a	Sheep PCC	Lys	Gly	Gln	Pro	Ile	Ala	Val	Leu	Ser	Ala	Met	BCT	Met	Gln	Asn	Ser	Met	Thr	Ala	Gly	Lys
b	Human PCC	Glu	Gly	Gln	Glu	Ile	Cys	Val	Ile	Glu	Ala	Met	BCT	Met	Gln	Asn	Ser	Met	Thr	Ala	Gly	Lys
c	Chicken PC		Gly	Ala	Pro	Leu	–	Val	Leu	Ser	Ala	Met	BCT	Met	Glu	Thr	Val	Val	Thr	Ala	Pro	Arg
c	Sheep PC		Gly	Gln	Pro	Leu	–	Val	Leu	Ser	Ala	Met	BCT	Met	Glu	Thr	Val	Val	Thr	Ser	Pro	Val
d	Human PC	Lys	Gly	Gln	Pro	Leu	Cys	Val	Leu	Ser	Ala	Met	BCT	Met	Glu	Thr	Val	Val	Thr	Ser	Pro	Met
e	Yeast PC	Lys	Gly	Gln	Pro	Val	Ala	Val	Leu	Ser	Ala	Met	BCT	Met	Glu	Met	Ile	Ile	Ser	Ser	Pro	Ser
f	Rat PC	Lys	Gly	Gln	Pro	Leu	Cys	Val	Leu	Ser	Ala	Met	BCT	Met	Glu	Thr	Val	Val	Thr	Ser	Pro	Met
g	E.coli ACC	Val	Gly	Asn	Thr	Leu	Cys	Ile	Val	Glu	Ala	Met	BCT	Met	Met	Asn	Gln	Ile	Glu	Ala	Asp	Lys
h	P.sherm.TC	Ala	Gly	Gln	Thr	Val	Leu	Val	Leu	Glu	Ala	Met	BCT	Met	Glu	Thr	Glu	Ile	Asn	Ala	Pro	Thr

a. Whittle (1986)
b. Lamhonwah et al. (1987)
c. Rylatt et al. (1977)
d. Freytag and Collier (1984)
e. Morris et al. (1987)
f. Cassady et al. (1987 a)
g. Wood and Zwolinski (1976)
h. Sutton et al. (1977)

BCT = Biocytin (the lysine residue to which the biotin is attached).

Table 1.3 Immunological Studies on Biotin Enzymes

KEY

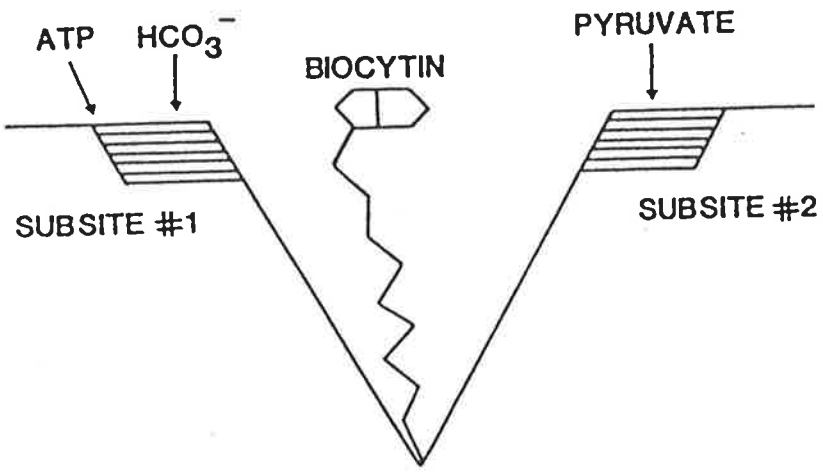
(a) Ballard et al. (1970)
(b) Sumper and Riepertinger (1972)
(c) Utter and Scrutton (1969)
(d) Majerus and Kilburn (1969)
(e) Mottershead et al. (1984)

PC	pyruvate carboxylase	I	inhibition of enzyme activity
PCC	propionyl-CoA carboxylase	O	no inhibition of enzyme activity
ACC	acetyl-CoA carboxylase	X	no cross reactivity by immunodiffusion techniques
TC	transcarboxylase		
S	small subunit of PCC	+	positive binding in ELISA
L	large subunit of PCC	-	negative binding in ELISA
d	denatured		

Table 1.3 Immunological Studies on Biotin Enzymes

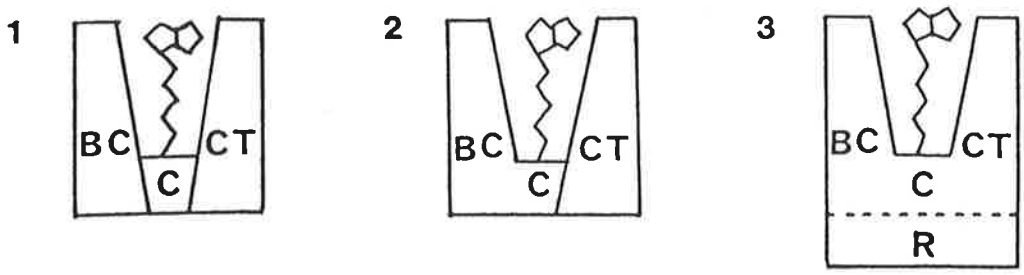
	Immunogen	Antigen Tested for Crossreactivity	Assay
(a)	PC (rat liver)	PC (in extracts of various rat tissues)	I
		PCC (rat liver)	O
		ACC (rat liver)	O
		PC (sheep liver)	I
(b)	ACC (yeast)	PC (yeast)	OX
	PC (yeast)	ACC (yeast)	OX
(c)	PC (chicken liver)	PC (turkey and chicken liver)	I
		PC (calf liver)	I
		PC (yeast)	O
		TC (*P. shermanii*)	O
	PC (yeast)	PC (calf liver)	O
		PC (turkey and chicken liver)	O
(d)	ACC (chicken liver)	ACC (rat liver)	I
(e)	dPC (chicken liver)	PC (chicken liver)	+
		$dPCC_S$ (sheep liver)	+
		$dPCC_L$ (sheep liver)	+
		PCC_{S+L} (sheep liver)	+
		ACC (chicken liver)	+
	$dPCC_L$ (sheep liver)	PC (chicken liver)	+
		$dPCC_S$ (sheep liver)	+
		$dPCC_L$ (sheep liver)	+
		PCC_{S+L} (sheep liver)	+
	$dPCC_S$ (sheep liver)	PC (chicken liver)	+I
		$dPCC_S$ (sheep liver)	+
		PCC_{S+L} (sheep liver)	+
		$dPCC_L$ (sheep liver)	-

FIGURE 1.1



Schematic representation of the proposed active site of pyruvate carboxylase depicting spatial separation of the two subsites, with the biocytin moiety free to oscillate between the two subsites. (Reproduced from Attwood and Keech (1984) with permission from the authors).

Figure 1.2 The Different Classes of the Biotin Enzymes



Type 1 organisation of the components of the active site is displayed by acetyl-CoA carboxylase from E. coli and Achromobacter and transcarboxylase from P. shermanii.

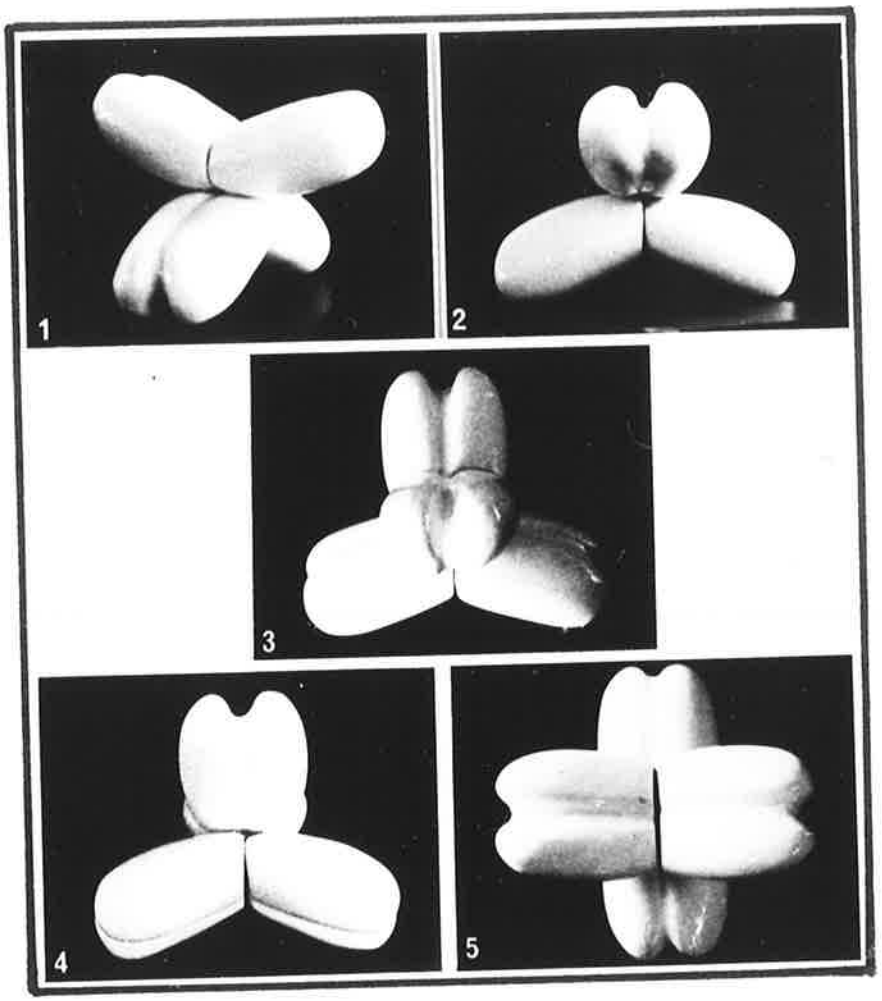
Type 2 organisation may be found in 3-methylcrotonyl-CoA carboxylase from Achromobacter.

Type 3 organisation is evident in the pyruvate carboxylases from sheep and chicken liver and yeast which contain sites for the binding of regulatory molecules.

R regulatory site
BC biotin carboxylase
C carboxyl carrier protein
CT carboxyl transferase.

Figure 1.3 Models of Sheep Liver Pyruvate Carboxylase Molecules Deduced from Electron Micrographs

These models show the appearance of SLPC which has been mounted in the presence of acetyl-CoA and viewed from different directions. Two pairs of subunits are oriented in different planes orthogonal to each other with opposing pairs of subunits contacting on their convex surface. A cleft is depicted running down the longitudinal midline of each subunit. The models show the intact SLPC tetramer. [Reproduced from Mayer et al. (1980) with permission from the authors].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CHAPTER 2

MATERIALS AND GENERAL METHODS

2.1 MATERIALS

2.1.1 Enzymes and Proteins

Albumin, bovine serum; glutamate dehydrogenase, type II, bovine liver (E.C. 1.4.1.3); glutamic-oxaloacetic transaminase, type I, porcine heart (E.C. 2.6.1.1.); malate dehydrogenase, porcine heart (E.C. 1.1.1.37); ovalbumin; papain, type IV, Papaya latex (E.C. 3.4.22.2); thyroblobulin, bovine; pyruvate kinase, yeast (E.C. 2.7.1.40); aldolase, rabbit muscle (E.C. 4.1.2.13); ferritin and avidin were supplied by Sigma Chemical Co., St. Louis, Mo., USA. Transcarboxylase, P. shermanii (E.C. 2.1.3.1) was a gift from Dr N. Phillips. Propionyl-CoA carboxylase, sheep liver (E.C. 6.4.1.3) was supplied by Dr G. Goodall and Ms J. Brazier. Pyruvate carboxylase, Saccharomyces cerevisiae (E.C. 6.4.1.1) was obtained from Mr F. Lim.

2.1.2 Radioactive Chemicals

Sodium [^{14}C] bicarbonate and d-[carbonyl^{14}C] biotin were supplied by The Radiochemical Centre, Amersham, England.

2.1.3 General Chemicals

ATP (disodium salt, grade I), CoA (grade I), dithioerythritol, NADH, trinitrobenzenesulphonate (TNBS), oxaloacetic acid, sodium pyruvate (type II, dimer free), d-biotin, L-lysine, guanidine-HCl (practical grade), EDTA (tetrasodium salt), SDS, Trisma base and O-tolidine dihyrochloride were obtained from Sigma Chemical Co., St. Louis, Mo., USA. Dithio-bis-[succinimidyl propionate] (DTSP) was obtained from

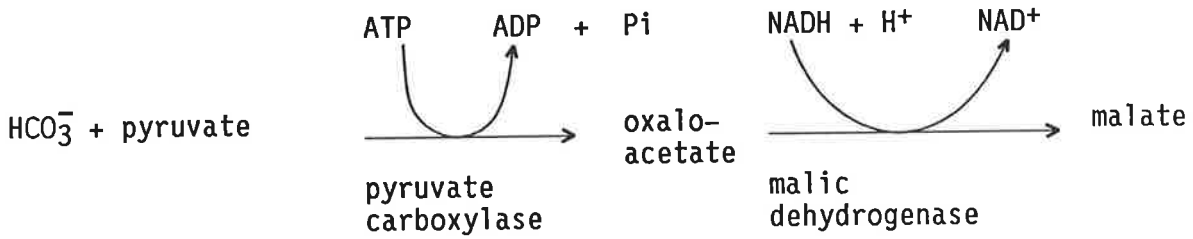
Pierce Chemical Company, Rockford, USA. N-ethyl maleimide (NEM) was obtained from Calbiochem (Aust) Pty Ltd, Sydney, Australia. Polyethylene glycol (molecular weight 20,000) was supplied by Union Carbide Corporation. Acrylamide, N,N-methylenebisacrylamide and N,N,N,N-tetramethylethylenediamine were obtained from Eastman Kodak, Rochester, New York, USA. Mann Research Laboratories, New York, USA supplied special enzyme grade ammonium sulphate. $MgCl_2$ was prepared from spec-pure magnesium (Hilger-Watts, Ltd, London, UK). Sucrose, analytical reagent grade, was obtained from Colonial Sugar Refining Co., Sydney, Australia. Triton X-100 was supplied by ICI (Aust) Ltd, Melbourne, Australia. POPOP [1,4-bis-2(4-methyl-5-phenoxazolyl)-benzene] and PPO (2,5-diphenyloxazole) were obtained from Koch-Light Laboratories Ltd, Bucks, England. Sephadex, Sepharose and Sephacel gel filtration and ion-exchange resins, cyanogen bromide activated Sepharose 4B and Protein A-Sepharose CL 4B were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. TSK G3000 and G4000 SW gel filtration columns were obtained from Beckman Instruments (Palo Alto, California, USA).

2.2 GENERAL METHODS

2.2.1 Acetyl-CoA dependent assay for pyruvate carboxylase

a) Spectrophotometric assay

This coupled assay is based on the procedure described by Utter and Keech (1963) whereby oxaloacetate produced by the pyruvate carboxylase reaction is reduced by excess malate dehydrogenase with the concomitant oxidation of NADH to NAD^+.



The NADH reduction is followed by absorbance at 340 nm using a Varian-Techtron 635-D spectrophotometer, thermostatted at 30°C. Activity was calculated using an extinction coefficient for NADH at 340 nm of 6.22 $mM^{-1}cm^{-1}$ where one unit of pyruvate carboxylase activity is defined as the amount of enzyme which carboxylates 1 µmole of pyruvate per minute in the assay solution described below.

The chicken liver pyruvate carboxylase assay solution contained 100 mM Tris-Cl pH 7.8, 2.5 mM ATP, 8 mM $MgCl_2$, 10 mM $NaHCO_3$, 10 mM sodium pyruvate, 250 µM acetyl-CoA, 125 µM NADH, 1 unit of malic dehydrogenase and 0.025 - 0.1 units of pyruvate carboxylase. The sheep liver pyruvate carboxylase assay solution was the same as above except that 100 mM Tris-Cl pH 8.4 was used instead of the pH 7.8 solution.

b) Radiochemical Assay

This is a direct assay which measures the amount of radioactivity incorporated into oxaloacetate from [^{14}C] bicarbonate. The components of the assay solutions are 100 mM Tris-Cl pH 8.4 (sheep assays) or pH 7.8 (chicken assays), 5 mM $MgCl_2$, 2.5 mM ATP, 10 mM sodium pyruvate, 10 mM $NaH^{14}CO_3$ (5-15 x 10^5 cpm/µmole), 250 µM acetyl-CoA and up to 0.05 units of pyruvate carboxylase. The reaction is initiated by the addition of the enzyme to the assay mix or vice versa and allowed to proceed for up to fifteen minutes at 30°C. The reaction is stopped by the addition of HCl to give a final concentration of 333 mM. This acidification also

drives off unreacted [^{14}C] bicarbonate. The oxaloacetate is stable under these conditions. Ten to twenty minutes after acidification, 50µl of the sample are spotted onto 2cm squares of Whatman 3MM paper, dried for 5 minutes at 105°C and counted in vials containing 3ml of toluene scintillation fluid. Although the spectrophotometric assay is more rapid, the radiochemical assay is more sensitive and was found to be more convenient for experiments involving a large number of assays.

2.2.2 Acetyl-CoA independent assay for sheep liver pyruvate carboxylase

This assay differs from the acetyl-CoA dependent radiochemical assay in that NH_4Cl is included in the assay solution and the concentration of pyruvate and bicarbonate is increased significantly. The components of the assay solution are 100 mM Tris-Cl pH 8.4, 8 mM $MgCl_2$, 2.5 mM ATP, 40 mM sodium pyruvate, 40 mM $NaH^{14}CO_3$ (5-15 x 10^5 cpm/µmole) and 100 mM NH_4Cl. The reaction is initiated by the addition of enzyme to the assay mix or vice versa and allowed to proceed for up to 10 minutes. The enzyme assayed must be at a concentration greater than 4 enzyme units/ml otherwise irreversible inactivation of the enzyme will occur. The reaction is terminated and processed as for the acetyl-CoA dependent radiochemical assay.

2.2.3 Assay for sheep liver propionyl-CoA carboxylase

Propionyl-CoA carboxylase catalyses the ATP dependent carboxylation of propionyl-CoA to form methyl-malonyl-CoA. Bicarbonate is the carboxyl group donor. To measure propionyl-CoA carboxylase activity, the incorporation of radioactivity from [^{14}C] bicarbonate into methyl-malonyl CoA is followed. The components of the assay solution are

4 mM ATP, 1.3 mM propionyl-CoA, 100 mM KCl, 10 mM $MgCl_2$, 1 mM DTE, 25 mM $NaH^{14}CO_3$ (5-15 x 10^5 cpm/μmole), 190 mM Tris-Cl pH 8.0 and up to 0.05 units of propionyl-CoA carboxylase. The reaction is initiated by the addition of the enzyme to the assay mix or vice versa and allowed to proceed for up to 15 minutes at 30°C. The reaction is stopped by the addition of trichloroacetic acid to 10% w/v final concentration. The radioactivity incorporated into methylmalonyl-CoA is determined as for the radiochemical assay of pyruvate carboxylase (2.1.1(b)).

2.2.4 Measurement of protein concentration

The concentration of protein in solutions containing enzymes was determined by the method of Layne (1957) whereby the absorbance of the solution at wavelengths of 260 and 280 nm for a 1cm light path was fitted to the formula:

$$mg/ml = 1.55A_{280\ nm} - 0.76A_{260\ nm}$$

The concentration of pure protein solutions was estimated by the method of Bradford (1976). For solutions of antibodies, the concentration was determined by the formula:

$$mg/ml = A_{280\ nm}/1.4$$ (Kabat and Mayer, 1961)

2.2.5 Preparation of acetyl-CoA

Acetyl-CoA was prepared by a similar method to that of Simon and Shemin (1953) and purified by the method of Keech and Barritt (1967).

2.2.6 Purification of pyruvate carboxylase

The purification of chicken and sheep liver pyruvate carboxylase was carried out according to Goss et al. (1979).

Freeze dried chicken or sheep liver mitochondria (120g) prepared by the method of Scrutton and Fung (1972) were suspended slowly into 1750ml of extraction buffer (25 mM Tris-acetate pH 6.7, 3.5 mM $MgCl_2$ and 1.7 mM ATP) and stirred for 20 minutes whilst maintaining the pH between 6.5 and 6.7. The suspension was centrifuged at 23000g for 20 minutes at 15°C to remove any undissolved material. The protein in the supernatant was precipitated by the addition of solid ammonium sulphate to give a final concentration of 1.35M.

a) Chicken liver pyruvate carboxylase

The ammonium sulphate precipitate was dissolved in Buffer A (25 mM potassium phosphate buffer pH 7.2, 20 mM ammonium sulphate, 1 mM EDTA, 0.1 mM dithioerythritol) to a final protein concentration of 10 mg/ml. This solution was desalted by the addition of finely ground solid polyethylene glycol (PEG) (14.5g/100ml) and stirring for 30 minutes whilst maintaining the pH at 6.9. The resulting precipitate was recovered by centrifugation (23000g for 15 minutes at 15°C) and dissolved in buffer A (0.5 ml/g of mitochondria extracted). A further centrifugation at 23000g for 15 minutes at 15°C removed any undissolved material and the supernatant was applied to a DEAE-Sepharose CL-6B column equilibrated in Buffer A. A linear gradient of ammonium sulphate from 20 mM to 200 mM in Buffer A was used to elute chicken liver pyruvate carboxylase.

b) Sheep liver pyruvate carboxylase

The precipitate resulting from the addition of ammonium sulphate to a final concentration of 1.35M was suspended in 1.07M ammonium sulphate pH 7.2 (0.5ml/g of mitochondria extracted). This back extraction was performed to reduce the level of glutamate dehydrogenase, often a major contaminant in these preparations. The precipitated protein was collected by centrifugation (23000g for 20 minutes at 15°C) and suspended in Buffer A to give a final protein concentration of approximately 10 mg/ml. Finely ground solid PEG was dissolved in this solution (14.5g/100ml) and the pH was adjusted to 6.7. This solution was stirred for 30 minutes following which the precipitated protein was collected by centrifugation (18000g for 15 minutes at 15°C). The precipitate was dissolved in a small amount of buffer A (0.5ml/g of mitochondria extracted). Any undissolved material was removed by a further centrifugation (18000g for 10 minutes at 15°C) and the supernatant was applied to a DEAE-Sephacel column equilibrated in buffer A. To elute pyruvate carboxylase, a linear gradient of ammonium sulphate from 20 mM in buffer A to 200 mM in 25 mM Tris-Cl pH 8.0, 1 mM EDTA, 0.1 mM dithioerythritol was employed.

The eluted pyruvate carboxylase from chicken or sheep liver was precipitated by ammonium sulphate at a final concentration of 1.85M. The protein was recovered by centrifugation at 24000g for 15 minutes at 15°C. The pellet was dissolved in 0.1M NEM-acetate, pH 7.2 containing 1.6M sucrose and stored at -80°C.

Pyruvate carboxylase from rat and kangaroo liver were prepared as for the sheep liver enzyme, however extraction with 1.07M ammonium sulphate was omitted when preparing the kangaroo enzyme. Pheasant and duck liver pyruvate carboxylase was prepared as for the chicken liver enzyme.

Using this method of purification, avian enzymes with specific activities of approximately 35-40 units/mg could be obtained. For the mammalian species enzymes with specific activities ranging from 20-25 units/mg were recovered (see Figure 3.3).

2.2.7 Avidin-Sepharose chromatography

a) Preparation of Avidin monomer-Sepharose column

Tetrameric avidin was coupled to cyanogen bromide activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) using the basic coupling procedure recommended by the suppliers of the gel. 3g of freeze-dried CNBr-activated Sepharose 4B was washed and reswollen on a sintered glass funnel with 600ml of 1 mM HCl. The gel was then washed rapidly with 150ml of coupling buffer (10 mM potassium phosphate pH 7.0, containing 0.5M NaCl) and added to coupling buffer containing 60mg of avidin. The volume of the gel, coupling buffer and avidin was 30ml. This solution was mixed end-over-end at 4°C for 16 hours. After filtering the gel to remove any unreacted avidin, the remaining reactive groups on the gel were blocked by the addition of 0.2M glycine pH 7.0 for 2 hours at room temperature with end-over-end mixing. Following this, the gel was washed with 400ml of coupling buffer. The gel was poured into a small column with a volume of 9ml. By measuring the absorbance at 280 nm of the avidin solution before and after coupling, it was determined that greater than 96% of the avidin was bound to the column.

The following procedures were carried out according to Henrikson et al. (1978). The avidin coupled to the column was dissociated into its monomeric form by washing the column with 50ml of 6M guanidine-HCl pH 4.8 then leaving for 16 hours at room temperature to assure complete

dissociation. Monomers of avidin have a reduced affinity for biotin and hence milder conditions for elution of a biotin enzyme from the column are available. The column was washed with a further 15ml of 6M guanidine-HCl and then with 100ml of coupling buffer until the absorbance at 280 nm was less than 0.01. To saturate residual tight biotin binding sites on the coupled avidin monomers, the column was washed with 35ml of 2 mM d-biotin. Biotin on the loose biotin binding sites was removed by a 60ml wash of 0.1M glycine-HCl pH 2.0. The column was equilibrated with 60ml of coupling buffer containing 0.1% w/v sodium azide. The biotin binding capacity of the column was determined using [^{14}C]biotin and was found to be 29 nmoles/ml of gel.

b) Purification of SLPC using avidin-Sepharose chromatography

When the specific activity of sheep liver pyruvate carboxylase prepared as described in section 2.1.4 was less than 20 units/mg, avidin-sepharose chromatography was used to remove contaminating protein and thus increase the specific activity of the enzyme.

SLPC (s.a. = 10 units/mg) was diluted two-fold into loading buffer (25 mM potassium phosphate pH 7.2 containing 1 mM EDTA and 0.1 mM dithiothreitol) taking precautions to ensure that the concentration of the enzyme was not lowered to less than 4 units/ml. Inactivation by dilution occurs when SLPC is less than 4 units/ml (Ashman et al., 1972). Routinely, 150 units of enzyme (approximately 38 pmoles of biotin) were applied to a 5ml Avidin-Sepharose column equilibrated in the loading buffer. The column was washed with the loading buffer until the absorbance at 280 nm was zero. Fractions from the wash were assayed for enzyme activity. Typically about 5% of the active enzyme did not bind to the column.

Pyruvate carboxylase was eluted from the column with a 25 mM potassium phosphate buffer pH 7.2 containing 1 mM EDTA, 0.1 mM dithiothreitol, 1 mM d-biotin, 200 mM KCl and 20 mM ammonium sulphate. The fractions containing active pyruvate carboxylase were pooled and precipitated by ammonium sulphate at a final concentration of 1.85M. The precipitated enzyme was recovered by centrifugation at 24000g for ten minutes as 15°C and solubilised either in a small amount (0.25 - 0.50ml) of enzyme storage buffer containing 0.1M NEM-acetate, pH 7.2 and 1.6M sucrose or in a suitable buffer (e.g. 0.1M Tris-Cl pH 7.2) if the enzyme was to be used immediately.

Using this method 85% of the active enzyme loaded was usually recovered from the column with a 2-3 fold increase in specific activity.

2.2.8 Determination of the concentration of biotin and biotin-binding sites

In avidin solutions the concentration of biotin-binding sites was determined by titration with [^{14}C]biotin as described by Rylatt et al. (1977). After digestion with proteases, the biotin content of pyruvate carboxylase solutions was also determined by this method.

2.2.9 SDS-polyacrylamide gel electrophoresis

Electrophoresis of protein was performed in a slab gel apparatus utilising the discontinuous system described by Laemmli (1970). The separating gels were typically 10% or 15% acrylamide and were stained with a solution containing 0.1% (w/v) Coomassie Blue R250, 50% (v/v) methanol and 10% (v/v) acetic acid. The gels were destained in a solution of 5% (v/v) methanol and 10% (v/v) acetic acid.

2.2.10 Enzyme linked immunosorbent assay (ELISA)

Both the urease and peroxidase assays are based on that of Engvall and Perlmann (1972) and were used to determine whether hybridoma supernatant, serum or ascites fluid contained antibodies which could bind to sheep liver pyruvate carboxylase or any other antigen tested.

a) Urease assay

Antigen to be coated onto the wells of a 96 well microtiter plate (Flexible polyvinyl chloride U bottom plates, Cooke Laboratory Products, Virginia, U.S.A.) was diluted to the appropriate concentration in 0.1M carbonate/bicarbonate buffer pH 9.6 containing 0.02% (w/v) sodium azide. Fifty µl of this antigen solution were pipetted into each well. The plate was covered and placed in a humid atmosphere at 37°C for one hour. The contents of the well were aspirated out and 200µl of an aqueous solution of BSA(1% w/v) were added to each well and incubated as for the antigen at 37°C for one hour. This saturates all remaining sites for protein binding in each well so that any binding of antibody in the next step is specific binding to the antigen. After the blocking step, the contents of the well were aspirated and the wells were washed three times with washing buffer (10 mM sodium phosphate, 150 mM sodium chloride pH 7.2 containing 0.05% v/v Tween 20). Fifty µl of the appropriate concentration of immune or pre-immune serum, ascites fluid or hybridoma cell culture supernatant (diluted if necessary in diluting buffer - 10 mM sodium phosphate, 150 mM NaCl, 0.25% BSA (w/v), 0.01% sodium azide (w/v) and 0.05% Tween 20, pH 7.2) were added to the wells and incubated at 37°C for 30 minutes under humid conditions. To remove any unbound antibody the solutions in the plates were aspirated and washed three times with washing buffer. After this, 50µl of rabbit-anti-mouse immunoglobulin conjugated to the enzyme urease (CSL,

Melbourne, Victoria) (1:100 dilution) were added to each well and allowed to incubate at 37°C for 30 minutes. To remove any unbound urease conjugated antibody, the plates were aspirated and washed three times with washing buffer then three times with twice distilled water. Following this, 50µl of undiluted urease substrate solution pH 4.8 (CSL, Melbourne, Victoria) were added to each well and incubated at 37°C for 30 minutes. The substrate solution consists of urea in a lightly buffered solution of the pH indicator bromocresol purple. In the presence of the enzyme urease, the urea in the substrate solution is hydrolysed to liberate ammonia and carbon dioxide. The released ammonia raises the pH of the solution resulting in a colour change of the pH indicator from yellow to purple. The reaction was stopped with 20µl of 35 µM mercuric chloride.

At the time of some of the early experiments there were no facilities available to measure the optical density of small volumes (50-100µl) such as there would be in the wells of a microtiter plate. Consequently the urease system was chosen over alkaline phosphatase and horseradish peroxidase systems because of its large colour difference between a positive and negative antibody reaction, ie. purple and yellow respectively. The visual endpoint is unequivocal and this enabled positive and negative wells to be recognised easily by eye. The intensity of the purple colour was graded from 0-4 where 0 represents a bright yellow colour, 4 represents a deep purple colour and 1, 2 and 3, while still positive, lie between the extremes of 0 and 4.

b) Peroxidase assay

This assay was carried out as described by Rylatt et al. (1983) with some modifications. The wells of a 96 well U bottomed microtiter plate

(Disposable Products Pty Ltd., Adelaide, South Australia) were coated with antigen by adding to each well 50µl of a 10µg/ml solution of the antigen in 137 mM NaCl, 5 mM KCl, 8 mM disodium hydrogen phosphate, 30 mM potassium di-hydrogen phosphate pH 7.2 (PBS). The plate was incubated for one hour at 37°C. Protein not bound to the plate was removed by inverting and tapping the plate. Non-specific binding sites were blocked by the addition of 0.3% gelatin in PBS and incubated for one hour at 37°C. The plates were then washed three times with PBS containing 0.05% Tween 20. Immune sera (50µl) diluted in PBS/Tween 20 was added to the wells and the plates were incubated for a further hour at 37°C. Unbound antibody was removed and the plates were washed as above. One hundred µl of a 1:1000 dilution of peroxidase conjugated rabbit anti-mouse immunoglobulin (Nordic, Tilburg, The Netherlands) in PBS/Tween 20 were added to the wells and the plate incubated for one hour at room temperature. Unbound conjugated antibody was removed and the plate was washed as before. Activated substrate (100µl) was added to each well. The substrate was activated immediately before use by adding 10µl of 3% H_2O_2 to 10ml of a solution containing 10 mM citrate, 2.5 mM O-tolidine dihydrochloride, 0.025 mM EDTA pH 4.5. The colour reaction was stopped after 10 minutes by the addition of 50µl of 3N HCl resulting in a colour change from blue to yellow for positives (negatives were clear). The absorbance was recorded at 450 nm on a Titertek Multiscan.

2.2.11 Competitive ELISA

This assay was a modification of the standard ELISA as described in Section 2.2.10(b). The aim of the assay was to determine whether two antibodies shared the same area of binding on SLPC.

The microtiter plate was coated for 1 hour at 37°C with 50µl of a 10µg/ml solution of either SLPC or SLPC modified with TNBS (in the presence or absence of 500 µM acetyl-CoA). The wells were subsequently blocked with 0.5% w/v gelatin for 1 hour at 37°C. After washing with PBS/0.05% Tween 20, appropriate dilutions of the two antibodies (or avidin) being tested were added in 50µl and incubated for 1 hour at room temperature. After incubation excess antibody was removed by washing with PBS/0.05% Tween 20. Appropriate dilutions of the two antibodies were determined as follows: the dilution of one of the antibodies was the lowest dilution able to produce an absorbance greater than 2.0 when tested alone against SLPC in a normal ELISA. This antibody was called the "challenging" antibody. Avidin was also used to challenge the test antibody at an avidin tetramer : SLPC subunit ratio of 2:1 (i.e. 585 ng; 8.6 pmole of avidin). The dilution of the other antibody to be used in the competitive ELISA was the lowest dilution which resulted in an absorbance of 1.0 when tested alone against SLPC. This antibody was termed the "test" antibody. It was necessary in this competitive assay that the two antibodies had been raised in different animal species because only the levels of "test" antibody bound to SLPC were quantitated by means of a third antibody of the correct specificity. The third antibody was conjugated to horseradish peroxidase and was directed towards mouse, rabbit or goat antibodies when the monoclonal antibodies, anti-TNP antibodies or anti-biotin antibodies respectively were used as 'test' antibody. The conjugated antibody (100µl) was incubated at room temperature for one hour. After washing as before, 100µl of substrate solution was added (see Section 3.2.17[b]) and colour development allowed for 10 minutes at room temperature. The reaction was stopped with HCl at a final concentration of 1M. The absorbance of each well was determined at 450 nm using a Titertek Multiscan plate reader.

2.2.12 Modification of sheep liver pyruvate carboxylase by trinitrobenzenesulphonate

Modification was carried out as described by Ashman et al. (1973). Sheep liver pyruvate carboxylase (26 units/mg) in 0.1M NEM-Cl pH 8.4 was modified by adding trinitrobenzenesulphonate (TNBS) (0.1 mM) at 30°C for 10 minutes. The modification was carried out both in the presence and absence of 500 µM acetyl-CoA. The reaction was stopped by quenching unreacted TNBS with L-lysine to a final concentration of 50 mM. At all times the enzyme concentration was greater than 4 units/ml to prevent inactivation by dilution.

2.2.13 Protein A - Sepharose chromatography

This chromatography was employed to purify IgG immunoglobulins from serum or ascites fluid. The procedure was carried out as described by Ey et al. (1978).

All chromatography was performed at 4°C. Staphylococcal protein A covalently linked to Sepharose CL-4B (Protein A-Sepharose, Pharmacia Fine Chemicals, Sweden) was swollen in 10 mM phosphate buffered saline (PBS) at pH 8.0 containing 0.1% w/v sodium azide. The gel was poured into a 5ml disposable syringe (fitted with gauze and O-rings) and equilibrated with the PBS. To 4-5ml of serum or ascites fluid, 2ml of 0.1M sodium phosphate pH 8.0 was added and the pH of this solution adjusted to pH 8.1. At this pH the IgG_1 subclass of antibodies will bind to protein A. The sample was loaded onto the column and then washed with 30ml of the pH 8.0 sodium phosphate buffer. Elution of IgG_1 antibody was achieved by passage of 30ml of 0.1M sodium citrate/citric acid pH 6.0

through the column. Subsequent washing with 30ml of 0.1M sodium citrate/citric acid pH 5.0 was optimal. To elute IgG_{2a} and IgG_{2b} antibodies 30ml of 0.1M citrate/citric buffer pH 4.5 and 3.0 respectively were passed through the column which was then re-equilibrated with the pH 8.0 phosphate buffer. Fractions of 3ml were collected. The IgG_{2a} and IgG_{2b} fractions were neutralised immediately with 1M Tris to prevent acid denaturation. The separation of the antibody subclasses from each other and from other serum proteins was monitored by measuring the absorbance at a wavelength of 280 nm.

2.2.14 Preparation of pure, denatured SLPC

To denature and ensure complete purity of SLPC, the enzyme was subject to electrophoresis on a 10% SDS polyacrylamide slab gel using the discontinuous system of Laemmli (1970). The protein bands were visualised after electrophoresis by soaking the gel in 10-12 volumes of 4M sodium acetate for 15-30 minutes (Higgins and Dahmus, 1979). The bands were cut out of the gel and soaked in 125 mM Tris-Cl pH 6.8, 0.1% (w/v) SDS, 1 mM EDTA, 5% (v/v) 2-mercaptoethanol for 15-30 minutes with occasional gentle shaking. The washed gel slices were then homogenised in 10ml of elution buffer (50 mM NEM-acetate pH 8.0, 0.1% (w/v) SDS, 5 mM EDTA, 150 mM NaCl) using a Potter Elvjhem homogeniser and left overnight at 4°C to elute. To remove the acrylamide, the homogenate was centrifuged at 23000g for 10 minutes at 5-10°C. The protein in the supernatant was precipitated by the addition of four volumes of cold acetone (-20°C) by holding the mixture in an ethanol/dry ice bath for 30 minutes. The protein was pelleted by centrifugation (23000g, 10 minutes, 4°C) and dissolved in 50 mM NEM-acetate pH 7.5, 1 mM EDTA, 1 mM DTE.

CHAPTER 3

PRODUCTION OF MONOCLONAL ANTIBODIES AGAINST SHEEP LIVER PYRUVATE CARBOXYLASE

3.1 INTRODUCTION

The work in this chapter describes the production of two sets of monoclonal antibodies directed against sheep liver pyruvate carboxylase. The purpose of this work was two-fold:

(1) It was considered an advantage to have monoclonal antibodies directed against SLPC. Those monoclonals which affected the activity of the enzyme would be a useful tool in studying the structure to function relationship of the enzyme. Conversely, those antibodies which did not affect enzyme activity, could be used in identifying areas on SLPC which are unimportant for the catalytic function of the enzyme. In addition, monoclonal antibodies could be used in the future to simplify the purification procedure for pyruvate carboxylase.

(2) Previous immunological studies on biotin enzymes have employed the use of polyclonal antibodies. Inhibition of enzyme activity, immunodiffusion and immunoprecipitation techniques using the polyclonal antibodies, have failed to show any cross reactivity amongst the different biotin enzymes tested (see section 1.3.4). In 1975, Lynen proposed that a family of biotin enzymes may have arisen from the duplication and fusion of the genes which encode the polypeptides of the active site (see section 1.3.1). Thus there has been considerable interest in analysing for immunological cross reactivity and hence structural similarity (at the antigenic determinants) amongst the biotin enzymes, in order to test Lynen's hypothesis.

Using the monoclonal antibody technique it was hoped that even infrequent clones producing cross reactive antibody could be isolated and amplified. To detect these clones an ELISA based system was used since it is known to be more sensitive than immunodiffusion and immunoprecipitation techniques.

3.2 METHODS

3.2.1 Immunisation Schedule for Mice to Provide Immune Serum and Immune Donor Spleen Cells

For this study female BALB/c mice were used. Before injection with the immunogen, each of the mice was bled to obtain pre-immune serum (see section 3.2.2.). The immunogen, sheep liver pyruvate carboxylase (SLPC) was relatively pure with a specific activity of 20-25 units per milligram. The mice were injected with the immunogen as follows:

(a) Injection 1

Sheep liver pyruvate carboxylase was diluted two fold into Freund's Complete Adjuvant and emulsified. The mice were injected subcutaneously with this emulsion such that 140µg of SLPC was injected per mouse (Week 0).

(b) Injection 2

At week 3 each mouse was injected intravenously with 60µg of an aqueous solution of SLPC. All of the mice were bled seven days later to obtain immune serum (see section 3.2.2.).

(c) Injection 3

(i) At week 16, two of the mice were boosted by intravenous injection of 100µg of an aqueous solution of SLPC. Four days after injection the mice were bled to obtain serum and their spleens were removed to investigate antibody production of immune spleen cells in culture (see section 3.3.1).

(ii) At week 18, two of the mice were boosted intravenously with 200μg of an aqueous solution of SLPC. Three days after injection the mice were bled for serum and their spleens removed for fusion with myeloma cells (see section 3.2.6).

3.2.2 Preparation of Mouse Serum From Blood

Immunised or non-immunised mice anaesthetised with ether were bled from the retro-orbital plexus. The blood was allowed to clot at room temperature for one hour and then at 4°C for 16 hours. The serum was drawn from the clot and spun at 23000g for ten minutes at 15°C to remove any red blood cells. It was made 0.1% w/v with respect to sodium azide and stored at 4°C.

3.2.3 Preparation of Spleen Cell Suspensions

The mice were killed and immersed briefly in ethanol. The remainder of this procedure was carried out in a sterile hood using sterile instruments and medium. The spleens were removed from the mice, freed of fat and cut into small pieces. These were homogenised by a loose fitting glass homogeniser using 10ml of serum-free medium (see 3.2.12) per spleen. The cell suspension was decanted away from any clumps of cellular debris and pelleted by spinning at 200g for five minutes. The cells were washed once in serum-free medium. The erythrocytes present were lysed by resuspending the cells in 5ml of tris-ammonium chloride and allowing to stand for ten minutes on ice. Tris-ammonium chloride for the red blood cell lysis is prepared by diluting aqueous ammonium chloride (0.83% w/v), ten times with 170 mM Tris-Cl pH 7.65. The cells were spun down as above, washed in serum-free medium and then resuspended in 10ml of serum-free medium.

3.2.4 Preparation of Parental Myeloma Cell Line for Fusion

Cells P3-NS-1-Ag4 (abbreviated NS-1, see Appendix B) in five 60ml flasks and in the log phase of growth, ie. 2-4 x 10^5 cells per millilitre were harvested into 50ml Falcon tubes. These cells were spun down at 200g for five minutes and washed once with approximately 30ml of serum-free medium. After resuspension in approximately 5ml of serum-free medium per Falcon tube, the tubes were pooled and a cell count made in a haemocytometer giving 12.5 x 10^7 cells in 25ml. The cell viability was greater than 95% as assessed by exclusion of 0.4% trypan blue.

3.2.5 Preparation of Immune Spleen Cells for Fusion

Two mice injected with SLPC in week 18 (see section 3.2.1.) were used for the fusion. Three days after boosting 0.5ml of blood was taken from each mouse and serum prepared. Their spleens were removed and a cell preparation was made as described in section 3.2.3. A total of 7.3 x 10^7 spleen cells in 10ml were obtained from Spleen 1 and 5.2 x 10^7 spleen cells in 10ml from Spleen 2.

3.2.6 Fusion of NS-1 Cells with Immune Spleen Cells

The procedure was carried out in a laminar flow cabinet. All reagents were warmed to 37°C prior to use. Before the fusion 2 grams of polyethyleneglycol (PEG), of approximate molecular weight 4000, was autoclaved and while still warm but not hot, 2ml of serum-free medium was added to the liquid PEG and mixed well. This solution was left at 37°C until required.

The NS-1 and spleen cells were pooled into Falcon tubes at a 1:1 ratio. For Spleen 1, 7.3 x 10^7 spleen cells in 10ml and 7.3 x 10^7 NS-1 cells in 14.6ml were pooled. For Spleen 2, 5.2 x 10^7 spleen cells in 10ml and 5.2 x 10^7 NS-1 cells in 10.4ml were pooled. The pooled cells were spun at 400g for five minutes. After removing all but about 1ml of the supernatant, the pellet was resuspended and the cells were spun again. As much of the supernatant as possible was then removed.

One ml of the warm 50% PEG (the fusing agent) was added to the NS-1/spleen cell pellet by slow dropwise addition over one minute, gently stirring the cell pellet with the tip of the pipette being used. Small clumps of cells were evident. The cells in PEG were incubated for a further two minutes at 37°C. This mixture was slowly diluted, while mixing, with 2ml of serum-free medium over two minutes at 37°C. A further 7ml of serum-free medium were added over 2-3 minutes. Slow addition of warm medium serves to gradually dilute the PEG without lysing the cells. This mixture was then centrifuged at 200g for five minutes, the supernatant removed and the cell pellet resuspended in HAT medium such that there are 2.08 x 10^6 spleen cells and 2.08 x 10^6 NS-1 cells per ml for each spleen.

This cell suspension was plated in 24 well plastic tissue culture plates (Costar #3524) and the plates were placed in a 37°C incubator humidified with 7% CO_2 in air. These plates were known as the master plates. The day of fusion was referred to as Day 0.

3.2.7 Growth of Mouse Hybrid Cells Secreting Antibody

Four days after the fusion, the cells in the 24 well trays were observed under an inversion phase contrast microscope to check for the appearance of hybrid cells and for contamination in the cultures. One ml of HAT medium was added to each well (see Appendix A for HAT selection of

hybrid cells). Hybrid cells were visible on Day 7. On Day 9, 1ml of medium was aspirated from each well and replaced with 1ml of fresh HAT medium. On Day 11 the hybrids in some wells were quite large and covered half to three-quarters of the wells. One hundred microlitres of medium was taken from each well and used to test by ELISA for the presence of antibodies in the medium against SLPC.

3.2.8 Expansion of Positive Wells to 6 Well Trays

Hybrids producing the relevant antibody were identified. Wells with ELISA readings of 2, 3 or 4 (see section 3.3.1.2) were expanded into 6 well trays (Costar) when the hybrids had covered at least half of the master plate well. The contents of the well (2ml) were resuspended and added to 3ml of HT medium in the 6 well trays. Three ml of HT medium were added back to the master plate since some cells still adhere to the bottom of the original well after transfer. It is necessary to grow the cells in HT medium, ie. no aminopterin for about one week after being in HAT medium, in order to dilute any remaining intracellular aminopterin. Between 2 and 5 days after this expansion, 5ml of HT medium were added to each cell.

3.2.9 Expansion to 50ml Dishes and Harvesting

When the hybrid cells were dense in the 6 well trays or the medium began to turn orange, the cells were resuspended and 7 of the 10ml in the 6 well trays were transferred to a 50ml Costar dish containing 20ml of fresh HT medium. Seven ml of fresh HT medium were added back to the original well. The cells were grown until the medium turned orange. The cultures were expanded by splitting in two and adding the same amount of fresh medium into each culture dish ('1 in 2' split). When the cells

were at a density of 4-5 x 10^5 cells per ml they were harvested into 50ml Falcon tubes. These cells were used for limit dilution cloning (see section 3.2.11) and cryopreservation (see section 3.2.10).

3.2.10 Cryopreservation of Cells

The viability of cells to be frozen was preferably greater than 90%. To cells at a concentration of 10^7-10^8 cells/ml in medium, an equal volume of "freezing mix" was added slowly with gentle shaking. Freezing mix consisted of 20% DMSO, 30% FCS and 50% RPMI [v/v]. The cells in freezing mix were pipetted into 1 or 2ml plastic screw cap vials (Nunc Intermed, Denmark) and frozen using a controlled rate freezer (Paton Industries, S. Australia) where the temperature dropped from ambient to 0°C at 5°C/minute, then from 0°C to -25°C at 1°C/minute and from -25°C to -100°C at 5°C/minute. After freezing the vials were stored in liquid nitrogen.

3.2.11 Limit Dilution Cloning of Hybridoma Cells

Limit dilution cloning was carried out as described by Oi and Herzenberg (1980). Since hybridoma cell densities are extremely low during cloning, "feeder cells" must be added. Thymocytes were chosen as the "feeder cells". Single cell suspensions of thymuses from BALB/c mice (4-10 weeks old) were prepared in HT medium such that the cell density was 10^7 cells/ml.

The hybrid cells used for cloning were 70-90% viable as assessed by trypan blue exclusion. Approximately 230 live cells in 100µl were added to 4.5ml of the thymocyte suspension and 100µl aliquots (5 cells/well) were dispersed into 36 wells of a 96 well microtiter plate. Four ml of thymocyte containing medium was added to the remaining 1 ml of cell

suspension and a further 36 wells were plated with 100µl aliquots (1 cell/well). This left 1.4ml of cell suspension to which 1.4ml of thymocyte suspension was added and used to plate the remaining 24 wells (0.5 cells/well). The plates were inculated at 37°C in a humidified atmosphere of CO_2 in air. One of these plating concentrations should yield a proper Poisson distribution of monoclonal growth.

Three to four days later 100µl of HT medium was added to each well. Every three to four days after this, half of the medium in each well was replaced with fresh HT medium. Ten days after cloning out, the wells were viewed under an inverting microscope to score for the presence of colonies of hybridoma cells. If less than 33% of the wells at a particular plating concentration contained growing hybridoma cells, then these wells were considered to contain monoclonal cultures. When there was confluent growth of the cells believed to be monoclonal, they were transferred to a 24 well plate where they were cultured in HT medium with thymocyte feeders. After a period of growth, medium from these cultures was tested for antibody binding to SLPC in the ELISA. Any positive clones were expanded as for the uncloned hybridomas. These cells were recloned once more, expanded and cryopreserved.

3.2.12 Tissue Culture Solutions

RPMI 1640 powder with added glutamine (two packets) was dissolved in 1.5 litres of water with 4g of $NaHCO_3$. The pH was adjusted to 7.4 with 1M HCl. HEPES pH 7.2, penicillin and streptomycin sulphate were added to give final concentrations of 15 mM, 500IU/ml and 100µg/ml respectively. The mixture, made up to two litres, was filter sterilised (0.2µm pore size) and 90ml aliquots were dispensed into sterile glass bottles. This filter-sterilised mixture was known as medium which was supplemented with 10% v/v foetal calf serum to support cell growth.

(Foetal calf serum was batch-tested for its ability to support the growth of P3 x 63Ag8.653 cells before use in hybridoma cell culture.) Glutamine to a final concentration of 2 mM was added to medium more than a week old. RPMI 1640 powder was obtained from Grand Island Biological Company, U.S.A., HEPES came from Calbiochem, while both penicillin and streptomycin sulphate were produced by Glaxo.

3.2.13 Thawing Frozen Cells

Frozen cells were removed from liquid nitrogen storage and thawed rapidly in a 37°C water bath. The contents of each vial was diluted slowly with an equal volume of medium at 37°C containing 10% FCS (v/v). After standing for fifteen minutes at 37°C, an equal volume of medium with 10% FCS (v/v) was added slowly and allowed to stand for a further 15 minutes. The cells were centrifuged at 200g for five minutes, resuspended in medium and washed twice.

3.2.14 Concentration of Culture Supernatants

Culture supernatants were concentrated ten fold using Centriflo CF-25 membrane cones (Amicon Corporation, U.S.A.) centrifuged at 754g in a Sorvall SS34 rotor for ten to fifteen minutes.

3.2.15 Ouchterlony Analysis of Antibody Class & Subclass

Ouchterlony plates were prepared by pipetting 1%(w/v) agarose in PBS/Azide pH 7.0 onto glass microscope slides (5 x 7.5cm) which had been pre-coated with 0.1% agarose in PBS/Azide pH 7.0. After setting, the plates were chilled for at least one hour at 4°C before cutting two sets of rosettes into each plate. Goat anti-mouse immunoglobulin (Meloy

Laboratories, Springfield) which reacted specifically with mouse IgG_1, IgG_{2a}, IgG_{2b} or IgM heavy chains, was pipetted into the central well of the rosette (10µl). The outer wells of the rosette were filled with 10µl of the serum or cell culture supernatant being tested. After incubation of the slides at 4°C for 48 hours in a humidified atmosphere, the slides were washed and stained for protein. To do this the slides were soaked in saline for 30 minutes then blotted to dryness with paper towels. This procedure was repeated five times, the last time soaking in distilled water overnight. To stain, the slides were incubated with 0.1% Coomassie Blue R250 in 5% methanol and 10% acetic acid for 30 minutes, then destained in 5% methanol and 10% acetic acid.

3.2.16 Preparation of Monoclonal Antibodies from Peritoneal Fluid

Mice were prepared 7-28 days before innoculation with hybridoma cells, by 0.2ml intraperitoneal injections of 'pristane' (2,6,10,14-tetramethyl pentadecane). Each mouse was then innoculated with 1-5 x 10^6 cells in serum-free medium. When ascites tumors were evident the mice were killed and the ascitic fluid was removed from the peritoneal cavity. The cavity was rinsed with 5ml of serum-free medium. The ascitic fluid was centrifuged at 200g for 5 minutes to remove hybrid cells. These cells were cryopreserved. The supernatant containing the antibodies was frozen at -20°C.

3.3 RESULTS AND DISCUSSION

3.3.1 Monoclonal Antibodies - Set A

This section reports the production of a set of monoclonal antibodies directed against sheep liver pyruvate carboxylase. At the time of this work, a tissue culture facility was not available in the Department of Biochemistry. Consequently this work was undertaken in the laboratory of Dr. Leonie Ashman of the Department of Microbiology and Immunology, University of Adelaide, under her guidance.

3.3.1.1 Propagation of Immune Spleen Cells in Culture to Monitor Antibody Secretion

After fusion of immune spleen cells with myeloma cells there is no positive selection against the growth of non-fused normal spleen cells and spleen-spleen cell hybrids in HAT medium (see Appendix A). However, passive selection takes place as normal spleen cells have limited growth potential under these culture conditions. Most spleen cells should die after two weeks in culture. Although each preparation of mouse immune spleen cells is different it was considered worthwhile to determine, for two immune mice, whether spleen cells in culture could pose problems after a fusion, giving rise to false positives in an ELISA screening for hybridoma originating antibody.

Spleens were removed from two immune mice (see section 3.2.1.[c]). A spleen cell preparation was made from each mouse as described in methods (see section 3.2.3) except that the cells were finally suspended into HAT medium containing 10% foetal calf serum. The cells from each spleen were plated out as for a "real" fusion with myeloma cells into 24 well trays. The cells were grown at 37°C in 5-7% humidified CO_2 as

described in Section 3.2.7. On days 7 and 10 live spleen cells were still present. A 50µl aliquot of cell culture medium was taken from 6 wells (where live cells were present) in each tray and pooled. An ELISA was carried out on the pooled culture supernatants from days 7 and 10 to determine whether antibodies directed against SLPC were present. In addition, immune sera from the two mice used for the spleen cell preparation as well as their pre-immune sera were included in the ELISA. The results are presented in Table 3.1. Clearly the two mice used for the spleen cell preparations were producing antibodies against SLPC (as detected in their serum). Therefore the spleens of these mice must have contained spleen cells producing antibodies against SLPC. These immune spleen cells in culture failed to give a positive reaction by ELISA at 7 and 10 days after plating out. This suggested that although some spleen cells still survived at days 7 and 10, the concentration of antibody in the culture medium was so low that no positive reaction was apparent by ELISA. Consequently, even though two different mice were used for the "real" fusion, it was expected that spleen cells fused or unfused living in culture would not give rise to false positive results when screening for antibody secreting hybridomas by this method.

3.3.1.2 Fusion

Mice were immunised with SLPC (see section 3.2.1.) and after the third immunisation, two mice were killed and single cell suspensions of their spleens were prepared (see section 3.2.5). These spleen cells were fused to P3-NS1-1-Ag4 myeloma cells using polyethylene glycol and plated out as described in section 3.2.6.

The sera from these mice were tested in ELISA for the presence of antibodies against SLPC (see Table 3.2). Clearly, the spleens of the two mice used in the fusion were producing antibodies which could bind to

SLPC where mouse 2 has the higher titre of antibodies in its serum. In addition, the sera from these mice were tested for their ability to inhibit SLPC activity. SLPC (150 mUnits in 5µl) was incubated with 10µl of serum for 1 hour at 30°C. After this, 0.05 units of enzyme was tested in the radiochemical enzyme assay for SLPC (see section 2.2.1(b)). Pooled pre-immune serum from these two mice and the immune serum used as a positive control in ELISA were also tested in this system. The degree of inhibition observed for the sera of the mice used for the fusion was the same as that for pre-immune serum. The serum used for the positive control in the ELISA was shown to inhibit 57% of enzyme activity which indicated that inhibition of enzyme activity was able to be observed in this system (results not presented). Thus it was concluded that the serum of the mice used for the fusion did not contain antibodies which inhibited enzyme activity.

Four days after fusion the cells were observed under an inversion phase contract microscope. A mixture of cells was observed which included small brown cells (dead spleen cells), large bright round refractive cells (healthy NS-1 cells), large brown cells (dead NS-1 cells) and large, bright, round, unvacuolated cells in clumps of 3 or 5 (hybridoma cells). On day 7 many hybrids were visible in the wells but none were big enough for assaying of their culture medium for the presence of antibodies against SLPC. Most NS-1 cells and many spleen cells were dead at this stage.

3.3.1.3 Initial Screening and Expansion

Eleven days after fusion, the hybrids in some wells were quite large. The medium was starting to yellow due to the presence of largely acidic metabolic waste products which affect the phenol red indicator. Aliquots (50µl) of cell culture medium were analysed by ELISA to

determine whether any hybridoma cells were producing antibody against SLPC. Culture medium from all 60 wells was tested in duplicate along with pre-immune serum pooled from the two mice used for the spleen cell preparations, mouse immune serum and cell culture medium in which cells were absent. The results of the ELISA are presented in Table 3.3.

Wells containing hybridomas producing antibody which resulted in a positive ELISA reading of 2, 3 or 4 were considered to be most promising and were expanded into 6 well trays as described in section 3.2.8. Those wells expanded were 1.1D5, 1.1D6, 1.1B2, 1.1C1, 1.1D4, 2D5 and 1.2D3.

When the cells in the 6 well tray were dense (preferably in the log phase of growth 2-4 x 10^5 cells/ml) they were expanded to 50ml Costar dishes as described in section 3.2.9.

Before cloning out, the cell cultures were tested for their continued ability to produce antibody which binds to SLPC in ELISA (see Table 3.4). Results from this ELISA indicated that all culture supernatants tested except for those from 1.1C1 and 1.1D6 still contained antibodies directed against SLPC. The ELISA was repeated twice and the same trends were observed. It was concluded that 1.1D6 and 1.1C1 were no longer producing antibodies against SLPC. Loss of antibody production can be due to chromosome loss, overgrowth of culture by non-antibody producing hybrid cells or overgrowth by hybrid cells producing antibody of another specificity. Since no antibodies against SLPC were detected for 1.1C1 and 1.1D6, it was concluded that the other cell cultures were truly positive since residual positive antibody in the cell medium from early cultures of 1.1C1 and 1.1D6 was not being detected to give false positive results.

3.3.1.4 Limit Dilution Cloning

It is possible that after a successful fusion as many as 600 hybrids may result. This means that if the fusion is plated out into two 24 well trays (as was the Spleen 1 fusion), then each well could contain about 12 different hybrids. The multiplicity of hybrids per well increases the chance of losing the desired secreting cell line by competition with other secreting or non-secreting hybrids. Therefore it is important when using 24 well trays after fusion, to clone out as soon as possible.

Samples of cell cultures 2D5, 1.1D5, 1.1D4, 1.2D3 and 1.1B2 were cryopreserved to protect against losing the new cell line while other samples of these cultures were cloned out by limit dilution cloning to obtain monoclonal cells (see section 3.2.11). For each of the five cell lines, none of the cloning dilutions exhibited monoclonal growth (results not shown). Nevertheless, a 50µl aliquot of cell culture fluid was taken from each well of the 96 well plates used for the cloning, and the aliquots within each row on the plate were pooled. The 8 pooled samples from each tray were tested in ELISA for their ability to bind to SLPC in order to select positives. After determining which rows of the 96 well tray were positive, one positive row (consisting of 12 wells) from each tray was expanded up to a 24 well tray. After the cells had reached confluent growth, the viable wells were tested by ELISA. Two or three positives from each row were chosen to be expanded into 6 well trays and grown. The positives chosen are listed in Table 3.5. They were subsequently cryopreserved before cloning out again.

The results of this second limit dilution cloning and the screening by ELISA of wells containing growing cells is presented in Table 3.5. Hybridoma 1.2D3 was not cloned out a second time.

Except for dilution 3 of 1.1B2/G12 and 1.1D5/A6 and dilution 2 of 2D5/C10, all growing cells exhibited monoclonal growth as described in Section 3.2.11. For the subclones of 1.1B2/G12, 1.1D5/A6, 1.1D5/A8, 2D5/C7 and 2D5/C10, between 25-100% of wells in which growing cells were present, did not produce antibodies able to bind to SLPC. This indicated that either the original positive wells contained a mixture of hybridomas or that non-antibody producing subclones had arisen. Since all subclones of 1.1D4/B8 and 1.1D4/B9 produced antibodies with the same reaction pattern as that of the original uncloned supernatant, the original positive well 1.1D4 probably contained monoclonal cells. Similarly 1.1B2/G8 was likely to have been monoclonal. Work with cell cultures 1.1B2/C9 and 2D5/C10 was discontinued since no growth after limit dilution of the cells was observed for the former and no growing cells producing antibody against SLPC was observed for the latter.

Table 3.6 lists the subclones believed to be monoclonal and able to produce antibodies against SLPC, that were expanded and cryopreserved. Before cryopreservation, cell culture supernatant from each subclone in the log phase of growth was collected. An aliquot of each cell culture supernatant was tested in ELISA for the continued ability to bind to SLPC. From Table 3.6 it is clear that all cell culture supernatants tested were positive. The remainder of the supernatants was concentrated 10 fold using centriflo CF-25 membrane cones (see section 3.2.14).

3.3.1.5 Ouchterlony Analysis of Antibody Class and Subclass

The antibody class and subclass were determined by Ouchterlony analysis where goat serum versus anti-mouse IgG and IgM heavy chains, γ and μ respectively were employed (see section 3.2.15). Standards used in the analysis were antibodies shown to have γ_1, γ_{2A}, γ_{2B} or μ heavy chains and were a kind gift from Dr. L. Ashman and Dr. S. Gadd.

The monoclonal antibodies will be referred to by the last letter and number of their final subclone code, eg. 2D5/C7/D5 will be referred to as D5.

In Figure 3.1(a) precipitin lines were observed between goat anti-mouse μ heavy chain serum and monoclonal antibodies H11, G9, D3, G6, H4, C5, H3, H4, E6, E8 and the IgM standard (contains μ heavy chains). Conversely antibodies D6, D5 and E10 do not exhibit precipitin lines. A different IgM standard was used in 3.1(a) with antibodies E6, E8 and D5 which gave a clearer precipitin line.

In Figure 3.1(b) precipitin lines were observed between goat anti-mouse γ_{2B} heavy chain serum and monoclonal antibodies D6, D5 and E10. Precipitin lines were not observed for antibodies H11, G9, D3, G6, C5, H3, H4, E6 and E8. The standard IgG_{2B} (contains γ_{2B} heavy chains) formed distinct precipitin lines.

When the monoclonal antibodies were tested against goat serum raised to γ_1 and γ_{2A} heavy chains, only the standards produced a precipitin line (not illustrated) indicating that none of the antibodies were of the IgG_1, or IgG_{2A} subclass. Table 3.7 summarises the results of the Ouchterlony analysis for antibody class and subclass.

The light chain (λ or κ chains) composition of the antibodies was not investigated since the antiserum against the light chains was not available. Hybridoma cells derived from NS-1 and spleen cells contain mixtures of the κ chains of the NS-1 cells and the λ and κ chains from the spleen cell immunoglobulin (see Appendix B). Since a high proportion of mouse immunoglobulins contain κ chains (S. Gadd, personal communication) it is likely that most of the monoclonals have light chains of the κ class.

3.3.1.6 Ouchterlony Analysis of Monoclonal Antibodies Against SLPC

Immunodiffusion plates were set up whereby sheep liver pyruvate carboxylase (specific activity 25 units/mg and 2.3mgs/ml) was placed in the central well of the rosette of an Ouchterlony plate and concentrated cell culture supernatants from the monoclonal cell lines were placed in the outer wells (see Figure 3.2).

From Figure 3.2 it was evident that not all of the uncomplexed protein had diffused from the gel before protein staining. Despite this high background, precipitin lines were observed for monoclonals D6, D5 and E10. For monoclonals antibodies tested with their homologous antigen, it would be expected that Ouchterlony analysis could reveal either a reaction of identity if the monoclonals were the same or a reaction of non-identity if the monoclonal antibodies were different. There would be no reaction of partial identity if the antibodies are truly monoclonal. Monoclonals D6, D5 and E10 form a contiguous precipitin line which indicates identity. Hence it is probable that D6, D5 and E10 are the same monoclonal antibody. This is possible since they were all derived from the same positive well from the first "cloning out".

None of the remaining monoclonal antibodies form precipitin lines. This does not indicate that antibodies directed against SLPC are not present. (ELISA results in Table 3.6 show that antibodies are present in these culture supernatants which bind to SLPC.) Three points to consider here are:

(i) for precipitation to occur optimum concentrations of antibody and antigen are required. Different concentrations of antibody in the immunodiffusion plates may have resulted in precipitation.

(ii) only if the antigenic determinant to which a monoclonal is made is represented more than once on a protein or if the protein consists of two or more subunits each containing the determinant, will cross linking by monoclonal antibodies occur that results in the formation of a 3-D lattice and precipitation. Since SLPC is composed of 4 apparently identical subunits, then crosslinking could occur provided that the antigenic determinant is located on an area of the subunit which allows greater than one antibody to bind per tetrameric molecule.

(iii) for crosslinking and precipitation to occur, both combining sites on the antibody must be functional. Fusion of a NS-1 cell with a mouse spleen cell may result in the production of three types of antibody: H_2L_2, H_2LK, H_2K_2 where H and L are the mouse antibody heavy and light chains respectively and K is the antibody light chain of the myeloma cell (see Appendix B). Both H_2L_2 and H_2LK have antigen binding capacity but only H_2L_2 is likely to crosslink different molecules of pyruvate carboxylase since it possesses two functional combining sites. (Heavy chains alone may bind to the corresponding antigen, though much less strongly than the combination of the heavy chain with its homologous light chain). The light chain composition of the antibodies was not determined however.

3.3.2 Monoclonal Antibodies - Set B

In this section the preparation of a second set of monoclonal antibodies versus sheep liver pyruvate carboxylase is reported. This work was done in collaboration with Dr. Dennis Rylatt of the Centre of Applied Immunology at the Queensland Institute of Technology, Brisbane, Queensland. Basically the workload was divided as follows: preparation

of sheep liver pyruvate carboxylase (SLPC) for use as an immunogen and screening for antibodies directed against SLPC (Adelaide), immunisation of mice, fusion and initial screening of hybridomas for cells producing antibodies which bind to SLPC (Brisbane), more refined screening including binding of antibodies to different biotin enzymes and inhibition of enzyme activity (Adelaide), expansion of cells, cloning out cells by limit dilution and preparation of ascites fluid containing monoclonal antibodies (Brisbane) and complete characterisation of antibodies (Adelaide) (see Chapters 4 and 5).

3.3.2.1 Preparation of SLPC for Immunisation and Use as an Antigen

Sheep liver pyruvate carboxylase was prepared as described in Section 2.2.6(b). The enzyme was of specific activity 8-10 units per milligram and was used to inject BALB/c mice. It was found to be an excellent immunogen with titres of 10^{-6} or greater by ELISA. (The titre was defined as being the largest dilution which gave $A_{450\ nm}$ of 0.1 above background.)

Pure SLPC both active and denatured, was prepared for subsequent screening of hybridomas. It was essential to screen the hybridomas using pure antigen since impure SLPC was used to immunise the mice from which the hybridomas were subsequently prepared.

Pure active SLPC was prepared by Avidin-Sepharose chromatography (see section 2.2.7(b)). SLPC of specific activity 22 units per milligram was obtained. Pure denatured enzyme was prepared from a SDS polyacrylamide gel as described in Section 2.2.14. Both pure denatured and active SLPC ran as single bands on an SDS polyacrylamide gel. See Figure 3.3(a).

3.3.2.2 Fusion and Initial Screening

Spleen cells from BALB/c mice injected with SLPC (see section 3.3.2.1) were used in the fusion with NS-1 myeloma cells. The fusion was carried out as described by Kelly et al. (1980) and plated out into 4, 96 well microtiter plates on Day 0. Seven to eight days later medium from the 384 wells were tested in an ELISA using the horseradish peroxidase system (see section 2.2.10[b]). This assay determined which cells were producing antibody able to bind to pure SLPC applied to microtiter plates in either an "active" or denatured form. It was understood that when active SLPC was bound to the microtiter plates, it was likely to be partially denatured in the process. All 384 wells tested, were positive for "active" SLPC and 32 of these were positive for denatured SLPC.

3.3.2.3 Secondary Screening

On Days 9, 10, 11, forty-seven wells containing the most useful antibodies were chosen to be expanded on the basis of results from (a) the ELISA assay where the antigen used to coat the wells of the microtiter plates were other biotin carboxylases, and, (b) inhibition of sheep liver pyruvate carboxylase activity by antibody in the medium.

(a) ELISA

For this section of work the urease ELISA system was utilised as described in section 2.2.10(a). The biotin carboxylases used to coat the 96 well microtiter plates were pyruvate carboxylase from sheep, chicken, pheasant, rat and kangaroo liver and propionyl-CoA carboxylase from sheep liver (see section 2.2.6 for preparation of biotin carboxylases). The amount of biotin enzyme present in each preparation was determined by

biotin assays (see section 2.2.8) and 50µl of a 100µg/ml solution of biotin enzyme were used to coat the microtiter plates irrespective of the overall protein concentration. Fig. 3.3(b) shows the other biotin enzymes which were used for the ELISA. These enzymes had not been further purified by Avidin-Sepharose chromatography. The pyruvate carboxylase band in each case lies in the 110-120 kDa region while the two subunits for sheep liver propionyl-CoA carboxylase have molecular masses of 58 and 72 kDa (Goodall et al., 1985). In all cases except for rat and pheasant pyruvate carboxylase, the band containing the biotin enzyme would represent more than 90% of the total protein. In the track containing the pheasant liver enzyme preparation, less than 50% of the total protein is contained in the pyruvate carboxylase band. The rat liver enzyme is also contaminated with other proteins. The purity of these other biotin enzymes in the ELISA was not a problem since the hybridomas which were positive had been screened with pure SLPC. Only if the contaminating proteins are antigenically related to SLPC will they pose a problem (eg. if other biotin enzymes have been co-purified with the ones in question here).

Due to the large number of culture supernatants which had to be screened over a short period of time and to the small amount of culture supernatant available, each biotin enzyme on the plate was only tested once for each culture supernatant. Fifty microlitres of a 100µg/ml solution of antigen were coated onto the microtiter plate and this was probed with 50µl of undiluted culture supernatant. Each microtiter plate used, contained a positive control where SLPC bound to the plate was probed with mouse anti-SLPC immune serum. Two negative controls were included. For one, SLPC was coated to the plate and probed with culture supernatant from hybrid cells producing antibody directed towards a protein unrelated to SLPC. For the other negative control the wells were coated only with the blocking agent, bovine serum albumin (BSA), and

probed with each culture supernatant. Wells which showed any development of purple colour were judged as being positive (ie. the antibody in the cell culture supernatant binds to the antigen coated on the microtiter plate). The two sets of negative control wells resulted in the substrate solution being yellow, ie. negative.

(b) Inhibition of SLPC Activity

To determine whether an antibody was able to inhibit SLPC activity, hybridoma culture supernatant was incubated with enzyme for one hour at 30°C. The activity of the enzyme was then measured in a radiochemical assay (see section 2.2.1(b)) and compared with the activity of enzyme which had been incubated with control culture supernatants under the same conditions. As for the ELISA, each culture supernatant could only be tested once in this assay due to the large number of samples and limitations in time and sample available.

The incubation mix comprised of 5µl of enzyme containing approximately 0.5 mUnits of enzyme, 5µl of 5 mM acetyl-CoA (330 µM final concentration) and 65µl of buffer or cell culture supernatant. At 0.5 mUnits of enzyme per 75µl incubation mix (ie. 6.7 mUnits/ml), SLPC is very susceptible to inactivation by dilution. This phenomenom is observed when enzyme is less than 4 units/ml. For SLPC 100µm acetyl-CoA is able to prevent dilution inactivation (Ashman et al. 1972). Hence, the presence of acetyl-CoA at a concentration of 330 µM in the incubation mix prevents inactivation from occurring. Assuming that an equimolar ratio of antibody of the IgG subclass (molecular mass 150 kDa) to active enzyme subunits (subunit molecular mass 117 kDa) is required to inhibit activity, 0.5 mUnits of enzyme (20 Units/mg) would require 30ng of antibody for inhibition. Antibody in culture supernatants is normally in the order of 10-60µg/ml (L. Ashman - personal communication). Hence for

the lower limit of this range, 3µl of culture supernatant would be required in this assay to observe inhibition of enzyme activity. In fact, 65µl of culture supernatant was used which would be expected to be at least a 22-fold excess of antibody required. It should be noted that the dissociation constant for the antibody and enzyme will have considerable bearing on the molar ratio of enzyme to antibody required to cause inactivation of enzyme activity.

After incubation of the enzyme with antibody, the enzyme activity was measured radiochemically (see section 2.2.1(b)). Since the amount of enzyme being assayed was 100 times less than usual, two alterations were made to the normal radiochemical assay to ensure the incorporation of measurable amounts of radioactive $^{14}CO_2$ into oxaloacetate. Firstly the specific activity of the $NaH^{14}CO_3$ was three times greater and secondly the assay time was extended to 15 minutes. The incorporation of $^{14}CO_2$ into oxaloacetate was shown to be linear for at least 15 minutes with 0.5-5.0 mUnits of SLPC. Hence substrates are not limiting in the time of the assay (data not presented). The activity of the enzyme incubated with each culture supernatant was expressed as a percentage of the activity of enzyme incubated with control culture supernatants.

From the 384 samples tested by ELISA and inhibition of SLPC (described above), 47 were chosen to be expanded from the 96 well trays to the 24 wells trays. Some of those chosen were selected to be either inhibitors or activators of enzyme activity, some which did not affect enzyme activity, some which bound to SLPC alone, a few which bound to all biotin enzymes tested, some which bound to all mammalian liver pyruvate carboxylases and others to all pyruvate carboxylases tested. Some of those chosen also bound to denatured SLPC. The properties of the 47

samples as determined by ELISA and inhibition of enzyme activity assays are summarised in Table 3.8 and are referred to as "PRE- EXPANSION". Each sample is identified by a clone number.

3.3.2.4 Expansion of Cells and Subsequent Screening

The cells from the specified 47 wells were expanded (see sections 3.2.8, 3.2.9) and frozen at the earliest possible time. Unfortunately cells from sample 44 became contaminated and were discarded. Cell culture supernatants were taken just before freezing the cells and the properties of these supernatants were again determined by ELISA with the different biotin carboxylases and denatured SLPC and by inhibition of activity of both pyruvate and propionyl-CoA carboxylase from sheep liver. Cell culture supernatants from C22.15.3B6 and C.22.15.4B2 were not tested.

(a) ELISA

The ELISA as described in Section 2.2.10(b) was carried out in duplicate. With the availability of a Titertek Multiscan plate reader, the absorbance of each well at a wavelength of 450 nm was determined. Figure 3.4 shows the average absorbance values for each of the 44 expanded samples tested against chicken, pheasant, rat, sheep and kangaroo liver pyruvate carboxylase and propionyl-CoA carboxylase. The mean of the control wells is indicated (n=6) as well as the mean plus two standard deviations. A positive was defined as having an absorbance at 450 nm which is greater than two standard deviations away from the mean of the absorbance of the negative controls. The positive or negative assignment for each of the samples, referred to as "EXPANDED", is shown

in Table 3.8. ELISA assays using denatured SLPC were also carried out on the expanded samples by the Queensland collaborators. The results are shown in Table 3.8.

(b) Inhibition of SLPC Activity

For the assays where inhibition of SLPC activity was being examined, each culture supernatant was tested on at least two separate occasions in triplicate while for SLPCC activity, each culture supernatant was tested at least once in triplicate. The value shown for the inhibition assays is the average of each result and is expressed as a percentage of the activity of enzyme which has been incubated with control culture supernatant. The results of these assays are shown in Table 3.8 where the samples are referred to as "EXPANDED".

(c) Inhibition of SLPCC Activity

The method of measuring sheep liver propionyl-CoA carboxylase activity is described in section 2.2.3. For the inhibition of enzyme activity assays, the 75µl sample consisted of 10µl of enzyme containing 0.5 mUnits of activity and 65µl of the cell culture supernatant being tested. Sheep liver propionyl-CoA carboxylase consists of two subunits of molecular masses 72 and 58 kDa, the former containing the biotin prosthetic group. These subunits are believed to be arranged in an $\alpha_6 \beta_6$ configuration (Goodall et al., 1985). Assuming that an equimolar ratio of antibody to either subunit is required to inhibit activity, then 0.5 mUnits of enzyme at 14 units/mg would require 41ng of antibody for inhibition. If the lowest concentration of antibody in cell culture supernatant is 10µg/ml, then 65µl of this would contain 650ng,

ie. at least a 16 fold excess of antibody is being used. Unlike SLPC, acetyl-CoA is not required for protection at high dilutions of the enzyme. The 75µl sample is incubated for one hour at 30°C and then assayed as described in Section 2.2.3.

(d) Discussion

From Table 3.8 it is clear that the properties of some of the samples have changed immensely upon expansion. Hybridoma cells are known to be relatively unstable soon after fusion with respect to both stability of their genetic material and the possibility of another cell line in the well outgrowing the cell line originally detected. Hence it was not a surprise to encounter these changes.

Considering the ELISA before and after expansion in the case where denatured SLPC was coated to the microtiter plates, the ability to react with denatured antigen was lost by C23.15.1A1, C24.15.2D4, C24.15.3D5 and C25.15.4B3, but gained by C22.15.3D1 and C23.15.1D1. For the ELISA using different biotin enzymes, the changes observed were usually loss of binding properties to some and even all biotin carboxylases in five cases. The ability to react with a biotin enzyme was gained however in some instances, eg. C.24.15.2D3 and C.24.15.4D6. For the inhibition assays the changes observed were quite large in many cases. There are no activators of enzyme activity after expansion. Because the properties of the "pre-expansion" wells have changed, only the properties of the expanded wells will be discussed in more detail.

Figure 3.5 summarises the results of the expanded samples. Inhibitors were defined as cell culture supernatants able to inhibit SLPC activity by 50% or more. Eleven samples were shown to be inhibitors of SLPC. Of those cell culture supernatants which were able to bind to SLPC (determined by ELISA), 89% also bind to rat liver pyruvate carboxylase,

68% of these bind to pyruvate carboxylase from kangaroo, 65% to chicken, 59% to pheasant and 24% of these bind to propionyl-CoA carboxylase. For this small sample at least, the rat enzyme appears to be, as would be expected, the most antigenically similar to the sheep liver pyruvate carboxylase. It was of interest that cross reaction was observed with the biotin enzyme, propionyl-CoA carboxylase. Ten of the samples were able to bind to SLPCC in ELISA. Figure 3.4 shows that C25.15.4A1, C25.15.4A2 and possibly C25.15.4A5 are borderline cases with respect to binding to SLPCC. Clearly C22.15.3B5, C22.15.3D1, C23.15.2D6, C24.15.2B1, C25.15.2D1, C25.15.4A3 and C25.15.4B3 are positive in their binding to SLPCC. At this time, immunological cross reaction amongst different biotin carboxylases had never been reported (see section 1.3.4). This provided some evidence that the different biotin enzymes tested in this system showed some structural similarity at least in their antigenic determinants. Only one sample inhibited enzyme activity by greater than 50%, C22.15.2D1. This sample was also able to inhibit SLPC activity and bound to all of the biotin carboxylases tested. Up to 33% inhibition of SLPCC activity was observed for three samples which were shown to be negative in their binding to SLPCC in the ELISA. A possible explanation for this observation is that determinants on the native enzyme which are essential for enzyme activity are not exposed when bound to a microtiter plate.

The biotin prosthetic group plays an essential role in the reaction which the biotin carboxylases catalyse. It was of interest therefore to determine whether any antibodies in the cell culture supernatants shown to inhibit enzyme activity were antibodies directed against the biotin prosthetic group. It was expected that if anti-biotin antibodies were present which inhibited activity, then the presence of excess biotin in solution would reverse this inhibitory effect.

(e) Effect of Biotin on Inhibition of Enzyme Activity by Cell Culture Supernatants

Sixty microlitres of cell culture supernatant were incubated with 5µl of 1 mM biotin in H_2O at pH 7.0 (5 nmoles) or 5µl of H_2O (neutralised with 1N NaOH) for 30 minutes at 30°C. If cell culture supernatant contains at most 60µg/ml of antibody of the IgG subclass (L. Ashman - personal communication) then 60µl would contain 24 pmoles of antibody. Hence, biotin (5000 pmoles in this assay) would be present in a vast excess.

After the pre-incubation of antibody with biotin or H_2O, 5µl of enzyme containing 0.5 mUnits of enzyme (SLPC, 20 units/mg) and 5µl of 5 mM acetyl-CoA were added and incubated for one hour at 30°C. Each cell culture supernatant was tested in this system in triplicate both in the presence and absence of biotin. The cell culture supernatants tested were those which had previously been shown to cause a 50% or greater inhibitory effect on the enzyme. The activity of the enzyme was determined as described in Section 2.2.1(b) and was expressed as a percentage of activity of the enzyme which was incubated with control culture supernatant both in the presence and absence of biotin. The presence of excess free biotin had previously been shown to have no effect on the apparent activity of enzyme in 0.1M Tris-Cl pH 7.2 or in control cell culture supernatant (data not presented).

Table 3.9 shows the results of this experiment. Unfortunately, at this time no anti-biotin antibodies (polyclonal or monoclonal) were available to use as a positive control, ie. reversal of the inhibitory effect of antibody on enzyme when excess biotin is present. However, even in the presence of a 208 fold excess of biotin over possible

anti-biotin antibodies, no complete reversal of inhibition of enzyme activity was observed, and it was concluded that none of the cell culture supernatants contained anti-biotin antibodies.

3.3.2.5 Cloning Out by Limit Dilution

Cell lines C.24.15.3C6, C.25.15.2B3, C.25.15.4A1 and C.25.15.4A5 failed to grow further. In addition it is evident from the ELISA results for the expanded samples that C.25.15.1D2, C.25.15.2B3, C.25.15.1C5, C.24.15.2A4 and C.24.15.1B6 have stopped producing antibodies against any biotin carboxylase tested. Hence 36 "cell lines" were producing antibodies at least against SLPC.

The eight samples chosen to be subcloned to ensure the production of monoclonal antibodies are indicated on Table 3.8 by °. Six of these were able to inhibit the activity of SLPC and one of these also inhibited SLPCC. Four bound to all of the biotin carboxylases tested, five bound to denatured SLPC and only one bound to SLPC (denatured or "active") alone. Until these cell lines were cloned out however, the properties of the cells were not considered to be completely stable. The eight "cell lines" were cloned out once by limit dilution as described in Section 3.2.11. The subclones available and the form of antibody provided are shown in Table 3.10. The full subclone number identifying each subclone is used in Table 3.10. Although this number gives the history of the clone, it is very lengthy. Consequently, from here on, the subclone number will be abbreviated to the number after the / in the full subclone number, e.g. C.25.15.4B3/42 will be referred to as 42 and C.24.15.4A1/6 will be referred to as 6. Note that C.25.15.2D1/12 will be known as 12 but C.25.15.4B3/12 will be known as 4B3/12 to avoid confusion.

These subclones were cryopreserved and are stored at the Centre of Applied Immunology of the Queensland Institute of Technology, Brisbane. Ascites fluids of 6, 12, 18, 42, 60, 78, 98 and 113 were provided and tested in an ELISA where both denatured and "active" SLPC were used to coat the plates. The ELISA employed is described in Section 2.2.10(b). The results are presented in Table 3.11.

All of the subclones except 78 and 80 have a reasonably high titre of antibodies in the ascites fluid directed against "active" SLPC coated to microtiter plates. For the four subclones tested against denatured SLPC, binding was evident but the titres were lower compared to those for "active" SLPC. The ability of these antibodies to bind to denatured SLPC was of interest for any future experiments involving probing denatured SLPC on nitrocellulose filters with these antibodies. It was understood however, that the form of the SLPC on the microtiter plate in ELISA and on a nitrocellulose filter was likely to be different.

In summary then, these subclones were selected to be monoclonal and the antibodies they produce were shown to bind to SLPC in ELISA. Hence, monoclonal antibodies directed against SLPC have been produced.

3.3.2.6 Purification of Monoclonal Antibodies by Protein A-Sepharose Chromatography

Monoclonal antibodies 6,12,18,42,60,78,80,98 and 113 in ascites fluid were purified by Protein A-Sepharose chromatography as described in Section 2.2.13. At this time the subclass of the monoclonal antibodies was unknown because antisera against the antibody subclasses was not available for Ouchterlony analysis. Ey et al. (1978) showed that with Protein A-Sepharose, mouse IgG subclasses could be fractionated from serum. The unadsorbed fraction contained IgM, IgA and IgE. IgG_1, IgG_{2a} and IgG_{2b} were eluted with buffers of pH 6.0-7.0, 4.5-5.0 and

3.5-4.0 respectively. Figure 3.6 shows the column profiles of each ascites fluid containing monoclonal antibody. After loading each ascites fluid onto the column in a 0.1M sodium phosphate buffer pH 8.1, a peak of protein was observed at a wavelength of 280 nm. With the subsequent elutions at pH 6.0, 5.5, 4.5 and 3.0, protein clearly eluted with the pH 6.0 buffer for ascites fluids containing antibodies 6,12,18,42,80 and 113. From the work of Ey et al. (1978) this suggested that these antibodies were of the IgG_1 subclass. Antibody 60 eluted from the Protein A column with the pH 3.0 buffer suggesting that this antibody was of the IgG_{2B} subclass. No protein eluted from the Protein A column below pH 8.0 for ascites fluids containing antibodies 78 and 98. This indicated that these antibodies may be IgM antibodies since IgM antibodies from mice do not bind to Protein A at the pH values tested here.

3.4 SUMMARY

The work in this chapter describes the production of two sets of monoclonal antibodies directed against sheep liver pyruvate carboxylase. To determine whether it was worthwhile to characterise these antibodies further, their ability to inhibit the activity of sheep and chicken liver pyruvate carboxylase was considered. Using the radiochemical assay as described in Section 3.3.2.3(b) no inhibition of enzyme activity was observed for Set A monoclonal antibodies in cell culture supernatants (data not presented). Before cloning out by limit dilution, several samples from Set B were shown to inhibit SLPC. Inhibition of propionyl-CoA carboxylase activity by some Set B samples was also observed. It was shown that none of these antibodies were directed at the biotin moiety. In addition, the Set B antibodies were screened on the basis of their binding to biotin carboxylases other than SLPC. Due to the

ability of some samples from Set B to inhibit SLPC activity as well as the added body of information on the binding to other biotin carboxylases, it was decided to pursue the further characterisation of the monoclonal antibodies produced in Set B. It was hoped that by investigating the binding of some monoclonal antibodies which inhibited enzyme activity, a greater understanding of the structure/function relationship of the enzyme would be achieved.

The Set A monoclonal antibodies were not characterised further. Nevertheless, the class and subclass of these antibodies was determined and although they are not inhibitors of enzyme activity, they may be useful in future structural studies. Epitope mapping of these antibodies on SLPC would be useful, at least to determine whether the antibodies within this group bind close to or at the same determinant.

Table 3.1 ELISA to Test for Antibody Production of Spleen Cells in Culture

Probe	Log_4 SLPC Dilution					
	0	-1	-2	-3	-4	-5
S1	4	4	3	0	0	0
S2	3	2	0	0	0	0
PI	0	0	0	0	0	0
I	4	4	4	3	0	0
C1/7	0	0	0	0	0	0
C2/7	0	0	0	0	0	0
C1/10	0	0	0	0	0	0
C2/10	0	0	0	0	0	0
C/0	0	0	0	0	0	0

The ELISA was carried out as described in section 2.2.10(a). The wells of a microtiter plate were coated with 50μl of SLPC of increasing four-fold dilution where undiluted SLPC was 100μg/ml. The SLPC was of specific activity 25 units/mg and had been purified by avidin-sepharose chromatography. The wells were probed subsequently with:

S1,S2 : immune sera from the two mice used for the spleen cell preparations. A ten-fold dilution of these sera was used.

PI : pre-immune sera pooled from the two mice used for the spleen cell preparations and diluted ten-fold.

I : mouse immune serum (see section 3.2.1[b]) diluted 100-fold.

C1/7,C1/10: culture supernatant from spleen 1 at days 7 and 10 used undiluted

C2/7,C2/10: culture supernatant from spleen 2 at days 7 and 10 used undiluted.

C/0 : cell culture medium.

The intensity of the final colour of the substrate solution was graded by eye from 0-4 where 0 (bright yellow) is negative and 4 (deep purple) is positive for antibodies against SLPC. Grades 1, 2 and 3 are positive as well but lie between the two extremes.

Table 3.2 ELISA to Test for Anti-SLPC Antibodies in the Serum of Two Mice Used as Spleen Donors for the Fusion to Myeloma Cells

	Log_4 SLPC Dilution					
Probe	0	-1	-2	-3	-4	-5
PI	0	0	0	0	0	0
I	4	4	4	3	0	0
S1	3	2	0	0	0	0
S2	4	3	2	2	0	0

The ELISA was carried out as described in section 2.2.10(a). The wells of a microtiter plate were coated with 50μl of SLPC of increasing four-fold dilution where undiluted SLPC was 100μg/ml. The SLPC was of specific activity 25 units/mg and had been purified by avidin-sepharose chromatography. The wells were probed with the following:

PI : pre-immune sera pooled from the two mice used for the spleen cell preparations and diluted ten-fold.

I : mouse immune serum (see section 3.2.1[b]) diluted 100-fold.

S1,S2 : sera from the two mice used as spleen donors for the fusion. These sera were diluted ten-fold.

The intensity of the colour of the substrate solution was grade by eye from 0-4 where 0 indicates a bright yellow colour (negative result) and 4 represents a deep purple colour (positive result). Grades 1, 2 and 3 are positive as well but lie between the two extremes.

Table 3.3 Initial Screening of Fusion by ELISA to Detect SLPC Antibodies

Sample	Colour Grade
1.2D3, Immune Serum	4
1.1B2, 1.1C1, 1.1D4, 2D5	3
1.1D5, 1.1D6	2
1.1B1, 1.1C2	1
Pre-immune serum, cell culture medium	0
Remainder of samples	0

The ELISA was carried out in duplicate as described in section 2.2.10(a). The wells of a microtiter plate were coated with 50μl of SLPC at 100μg/ml. The SLPC was of specific activity 30 units/mg and had been purified by avidin-sepharose chromatography. The wells were probed subsequently with:

1. mouse immune serum (see section 3.2.1[b]) diluted 100-fold.
2. cell culture medium from the 60 wells of the fusate used undiluted. The sample nomenclature is as follows:
   the first two numbers denote the spleen and tray number respectively, the letter represents the tray row and the last digit represents the tray column.
3. pre-immune sera pooled from the two mice used for the fusion diluted ten-fold.
4. cell culture medium used undiluted.

The intensity of the final colour of the substrate solution was graded by eye from 0-4 where 0 is a negative (bright yellow) result and 4 is the strongest positive result for antibodies against SLPC (purple). Grades 1, 2 and 3 are positive also and lie between the two extremes.

Table 3.4 ELISA to Test for Presence of Antibodies Against SLPC in Cell Culture Supernatants Before Cloning Out

The ELISA was carried out as described in section 2.2.10(a). The wells of a microtiter plate were coated with 50μl of SLPC (purified by avidin-sepharose chromatography and of specific activity 30 units/mg) of increasing 2-fold dilution where undiluted SLPC is 100μg/ml.

The wells were probed with the following:

I : mouse immune serum (see section 3.2.1[b]) diluted 100-fold.

2D5, 1.1D6, 1.1D5, 1.1D4, 1.2D3, 1.1C1 and 1.1B2 :
cell culture supernatants of cultures from 50ml tissue culture dishes.

PI : pre-immune sera pooled from mice used for the spleen cell preparations and diluted ten-fold.

C : cell culture supernatant from cells producing antibody against an unrelated antigen.

The intensity of the final colour of the substrate solution was graded by eye from 0-4 where 0 (bright yellow) is negative and 4 is the strongest positive result (purple) for antibodies against SLPC. Grades 1, 2 and 3 are positive as well but lie between the two extremes. Some dilutions were not tested (-).

Table 3.4

Probe	Log_2 SLPC dilution											
	0	-1	-2	-3	-4	-5	-6	-7	-8	-9	-10	-11
I	4	4	3	3	0	0	-	-	-	-	-	-
2D5	4	3	2	0	0	0	0	0	0	0	0	0
1.1D6	0	0	0	0	0	0	0	0	0	0	0	0
1.1D5	3	2	0	0	0	0	0	0	0	0	0	0
1.1D4	4	4	4	4	4	3	3	3	3	0	0	0
1.2D3	2	2	2	2	0	0	0	0	0	0	0	0
1.1C1	0	0	0	0	0	0	0	0	0	0	0	0
1.1B2	3	2	0	0	0	0	0	0	0	0	0	0
PI	0	0	0	0	0	0	-	-	-	-	-	-
C	0	0	0	0	0	0	-	-	-	-	-	-

Table 3.5 Limit Dilution Cloning

Limit dilution cloning was performed on each of the 5 hybridomas listed. On each cloning tray, none of the dilutions of cells exhibited a Poisson distribution of monoclonal growth (results not shown). An ELISA was performed to determine which wells from the first cloning contained anti-SLPC antibodies. Two or three positives from each cloning tray were chosen to be expanded and cloned again (listed in table). DILN 1, 2 and 3 are the three cloning dilutions , viz. 5 cells/well, 1 cell/well, 0.5 cells/well (see section 3.2.11), used on each tray of the second cloning. Wells which contained growing cells for each dilution and those which are able to produce anti-SLPC antibodies (determined by ELISA) are shown in the table. Only those dilutions in which one-third or less of the wells contained growing cells were considered to be monoclonal.

* Indicates wells not considered to be monoclonal.

Table 3.5 Results from Limit Dilution Cloning

Hybridoma	Positive Wells Chosen From 1st Cloning	Proportion of Wells Containing Growing Cells (2nd Cloning)			Proportion of SLPC Producing Clones
		DILN 1	DILN 2	DILN 3	
1.1B2	C9	0/36	0/36	0/24	0
	G8	0/36	0/36	6/24	6/6
	G12	0/36	0/36	10/24*	1/10
1.1D4	B8	1/36	0/36	0/24	1/1
	B9	0/36	8/36	5/24	13/13
1.1D5	A6	0/36	0/36	10/24*	1/10
	A8	0/36	11/36	1/24	9/12
1.2D3	None	-	-	-	
	C7	0/36	7/36	3/24	4/10
2D5	C10	0/36	13/36*	1/24	0/14

Table 3.6 ELISA to Test for Anti-SLPC Antibodies in Monoclonal Cell Culture Supernatants

The ELISA was carried out as described in section 2.2.10.(a) The wells of a microtiter plate were coated with 50μl of SLPC (purified by avidin-sepharose chromatography and of specific activity 30 units/mg) of increasing two-fold dilution where undiluted SLPC is 100μg/ml. The wells were probed with the following:

1. cell culture supernatants of monoclonal subclones (listed on table) from 50ml tissue culture dishes just before harvesting and cryopreservation.
2. mouse immune serum (see section 3.2.1[b]) diluted 100-fold.
3. pre-immune sera pooled from mice used for the spleen cell preparations and diluted ten-fold.
4. cell culture supernatant from cells producing antibody against an unrelated antigen (control).

The intensity of the final colour of the substrate solution was graded by eye from 0-4 where 0 (bright yellow) is negative and 4 if the strongest positive result (purple) for antibodies against SLPC. Grades 1, 2 and 3 are positive as well but lie between the two extremes.

Table 3.6

Probe	Log_2 SLPC Dilution 0	-1	-2
1.1B2/G8/G9	2	1	0
1.1B2/B8/G6	2	1	0
1.1D4.B7.C5	4	4	3
1.1D4/B9/H4	4	4	4
1.1D4/B9/H11	4	4	4
1.1D4/B9/H3	4	4	4
1.1D5/A8/E6	4	3	2
1.1D5/A8/E8	4	3	2
1.1D5/A8/D3	4	3	2
2D5/C7/D5	4	4	4
2D5/C7/E10	4	4	4
2D5/C7/D6	4	4	4
Immune	4	4	4
Pre-Immune	0	0	0
Control	0	0	0

Table 3.7 Class and Subclass of Monoclonal Antibodies

Monoclonal Antibody	Antibody Class/Subclass
1.1D4/B9/H11	IgM
1.1B2/G8/G9	IgM
2D5/C7/D5	IgG_{2B}
1.1D5/A8/D3	IgM
1.1B2/G8/G6	IgM
1.1D4/B9/H4	IgM
2D5/C7/D6	IgG_{2B}
2D5/C7/E10	IgG_{2B}
1.1D4/B8/C5	IgM
1.1D4/B9/H3	IgM
1.1D5/A8/E6	IgM
1.1D5/A8/E8	IgM

Table 3.8 Summary of ELISA and Inhibition of Enzyme Activity Assays After Secondary Screening

Table 3.8 summarises the ELISA and inhibition of enzyme activity assays performed on the cell culture supernatants of the 47 clones chosen to work with after the secondary screening. The properties of these clones before and after expansion are shown. The binding of each sample to different biotin enzymes was investigated using ELISA assays. The ELISA performed with the pre-expanded samples was carried out using the urease-based ELISA as described in section 2.2.10(a). Positive binding was determined by eye. Any development of purple colour was designated positive. The ELISA performed with the expanded samples was carried out using the peroxidase-based ELISA as described in section 2.2.10(b). Positive binding was defined as having an absorbance at 450 nm which was greater than two standard deviations above the mean absorbance of a negative control. The inhibition of SLPC activity by both the pre-expanded and expanded samples is shown. Inhibition of SLPCC activity by the expanded samples was also investigated. The % activity was defined as being the average activity of the enzyme after incubation with the cell culture supernatant expressed as a percentage of the activity of enzyme incubated with control culture supernatants. For pre-expansion samples n = 1, for expanded samples n = 6 for SLPC and n = 3 for SLPCC. The eight samples chosen to be cloned out by limit dilution are indicated by °.

Table 3.8

SAMPLE (CLONE KEY)	ELISA PRE-EXP S_D	C	P	R	S	K	PCC	ELISA EXPANDED S_D	C	P	R	S	K	PCC	% ACTIVITY PRE-EXP S	% ACTIVITY EXPANDED S	% ACTIVITY EXPANDED PCC
C22.15.1B4°	+	+	+	+	+	+	+	+	−	−	−	+	−	−	86	20	85
44	+	+	+	+	+	+	+				*				100	*	*
C.22.15.3B5	−	+	+	+	+	+	+	−	+	+	+	+	+	+	100	27	84
3B6	−	+	+	+	+	+	−				NT				118	NT	NT
3D1°	−	+	+	+	+	+	+	+	+	+	+	+	+	+	103	3	76
3D2	+	+	+	+	+	+	−	+	+	−	+	+	+	−	101	88	81
4B2	−	+	+	+	+	+	+				NT				124	NT	NT
4C3	+	+	+	+	+	+	−	+	+	+	+	+	+	−	83	94	82
4C4	−	+	+	+	+	+	−	−	+	+	+	+	+	−	76	68	76
C.23.15.1A1	+	−	−	+	+	+	−	−	+	+	+	+	+	−	86	73	76
1C5	−	+	+	+	+	+	+	−	−	−	−	−	−	−	127	103	69
1C6	+	+	+	+	+	+	+	+	±	+	+	+	−	−	117	83	70
1D1	−	+	+	+	+	+	−	+	−	−	−	±	−	−	77	92	65
2A6	−	+	+	+	+	+	−	−	−	−	+	+	−	−	105	89	67
2D6	−	+	+	+	+	+	+	−	+	+	+	+	+	+	96	17	85
3A2	−	+	+	+	+	+	−	−	+	+	+	+	+	−	3.6	101	133
3B1	−	+	+	+	+	+	−	−	+	+	+	+	−	−	79	66	79
3B2	+	+	+	+	+	+	−	+	−	+	+	+	+	−	86	31	67
3C4	−	+	+	+	+	+	−	−	−	−	+	+	+	−	69	87	91
4A6	+	+	+	+	+	+	−	+	+	+	+	+	+	−	89	91	83
C.24.15.1B6	−	+	+	+	+	+	−	−	−	−	−	−	−	−	127	101	89
1C1	−	+	+	+	+	+	−	−	+	+	+	+	+	−	126	48	71
1D1	−	−	−	+	+	−	−	−	−	−	−	+	−	−	98	98	76
1D5	−	+	+	+	+	+	−	−	−	−	+	+	−	−	132	101	78
1D6	+	+	+	+	+	−	−	+	+	+	+	+	−	−	129	107	80
2A4	−	−	−	+	−	−	−	−	−	−	−	−	−	−	175	108	80
2B1	−	+	+	+	+	+	−	−	+	+	+	+	+	+	155	104	99
2B2	−	+	+	+	+	+	−	−	−	−	+	+	−	−	85	81	92

Table 3.8 (cont.)

SAMPLE (CLONE KEY)	ELISA PRE-EXP S_D	C	P	R	S	K	PCC	ELISA EXPANDED S_D	C	P	R	S	K	PCC	% ACTIVITY PRE-EXP S	% ACTIVITY EXPANDED S	% ACTIVITY EXPANDED PCC
C.24.15.2D3°	–	–	–	+	+	–	–	–	+	–	+	+	+	–	175	9	103
2D4°	+	+	+	+	+	+	+	–	–	–	+	+	–	–	172	96	86
3C6	–	–	–	+	+	+	–	–	–	–	+	+	+	–	54	91	86
3D3	–	+	+	+	+	+	+	–	–	–	+	+	–	–	64	92	86
3D5	+	–	–	+	+	+	–	–	–	–	–	+	–	–	64	9	97
4A1°	+	–	–	+	+	–	–	+	–	–	+	+	–	–	63	0.4	88
4D6	–	–	–	+	+	–	–	–	+	+	+	+	+	–	60	68	114
C.25.15.1D2	–	+	+	+	+	+	+	–	–	–	–	–	–	–	92	95	87
2B3	–	–	–	+	+	+	–	–	–	–	–	–	–	–	39	97	50
2B5	–	+	+	+	+	+	+	–	+	+	+	+	+	–	34	101	109
2D1°	+	+	+	+	+	+	+	+	+	+	+	+	+	+	46	22	37
4A1	–	–	–	+	+	+	–	–	+	+	+	+	+	±	56	95	100
4A2	–	–	–	+	+	+	–	–	–	–	+	+	±	±	59	99	103
4A3°	+	+	+	+	+	+	–	+	+	+	+	+	+	+	61	98	105
4A5	–	+	+	+	+	+	–	–	+	+	+	+	+	+	65	97	96
4B2	+	+	+	+	+	+	–	+	+	+	+	+	+	–	61	100	98
4B3°	+	+	+	+	+	+	–	–	+	+	+	+	+	+	62	35	100
4B5	+	–	–	+	+	–	–	+	+	+	+	+	–	–	64	109	101
4C4	–	+	+	+	+	+	–	–	+	+	+	+	+	–	50	97	97

KEY

S_D	sheep liver pyruvate carboxylase (denatured)
S	sheep liver pyruvate carboxylase
C	chicken liver pyruvate carboxylase
P	pheasant liver pyruvate carboxylase
R	rat liver pyruvate carboxylase
K	kangaroo liver pyruvate carboxylase
PCC	sheep liver propionyl-CoA carboxylase
PRE-EXP	Cell culture supernatants before initial expansion
EXPANDED	Cell Culture supernatants after initial expansion
*	Cell line lost
NT	Not tested
+	Positive binding
–	Negative binding
±	Borderline result
o	Samples chosen to be cloned out by limit dilution

Table 3.9 Determination of presence of anti-biotin antibodies

Sample (Clone Key)	% Activity (+) Biotin	% Activity (-) Biotin
C.22.15.1B4	28.9 ± 0.8	30.7 ± 0.6
C.22.15.3B5	35.3 ± 1.5	33.6 ± 1.0
C.22.15.3D1	4.3 ± 1.2	6.3 ± 1.6
C.23.15.2D6	17.7 ± 0.8	14.8 ± 0.7
C.23.15.3B2	34.9 ± 0.3	34.4 ± 2.0
C.24.15.1C1	62.6 ± 4.1	69.8 ± 2.3
C.24.15.2D3	11.8 ± 6.8	13.5 ± 3.9
C.24.15.3D5	9.4 ± 1.0	8.8 ± 1.0
C.24.15.4A1	0	0
C.25.15.2D1	28.3 ± 5.2	29.5 ± 0.7
C.25.15.4B3	44.1 ± 11.0	48.2 ± 6.2

Cell culture supernatant (60 μl) from each sample shown to inhibit SLPC activity by 50% or more were incubated with 5 μl of 1 mM biotin (+) or H_2O (-) for 30 minutes at 30°C. Acetyl-CoA (5 μl of 5mM) and SLPC (0.5 mUnits, 20 units/mg in 5 μl) were added and incubated for a further hour at 30°C. The activity of the enzyme was determined radiochemically as described in section 2.2.1(b) and is expressed as a percentage of the activity of the enzyme in control culture supernatant ± the standard error of the mean (n = 3).

Table 3.10 Monoclonal Cells and Antibodies Available

Subclone No.	Subclone No. (abbrev.)	Form Available	
C.22.15.1B4/113	113	A	S
C.22.15.1B4/125	125		S
C.22.15.3D1/18	18	A	
C.22.15.3D1/60	60	A	
C.24.15.2D3/48	48		S
C.24.15.2D3/98	98	A	S
C.24.15.2D4/80	80	A	S
C.24.15.2D4/97	97		S
C.24.15.4A1/6	6	A	
C.24.15.4A1/57	57	-	
C.24.15.4A1/92	92	-	
C.25.15.2D1/12	12	A	
C.25.15.2D1/170	170	-	
C.25.15.4A3/61	61	-	
C.25.15.4A3/78	78	A	
C.25.15.4B3/42	42	A	S
C.25.15.4B3/16	16		S
C.25.15.4B3/12	4B3/12		S *
C.25.15.4B3/83	83		S *

* = Cell Line No Longer Available

Table 3.10 shows the monoclonal cells which produce antibodies against SLPC. These cells are stored at the Centre of Applied Immunology at the Queensland Institute of Technology, Brisbane. The form of the monoclonal antibody supplied i.e. in ascites form (A) or contained in cell culture medium (S) is also indicated.

Table 3.11 ELISA to determine titre of antibody in ascites fluid

	Titre*	
Subclone	"Active"	"Denatured"
6	5×6^6	1×10^4
12	2×10^7	6.4×10^5
18	1×10^6	NT
42	2×10^6	NT
60	1×10^6	1×10^4
78	6.4×10^3	1×10^3
80	8×10^3	NT
98	1.3×10^5	NT
113	1×10^6	NT

SLPC (denatured and active) was coated to microtiter plates and probed with ascites fluid of the subclones indicated in the table. The ELISA was carried out as described in section 2.2.10(b). The titre* was defined as being the highest dilution which gave an A_{450nm} of 0.1 above background and is expressed as the reciprocal of that dilution.

NT = not tested

Figure 3.1 Ouchterlony Analysis of Monoclonal Antibody Class and Subclass

(a) The central well of each rosette contains goat serum against mouse μ chain (10μl). Ten μl aliquots of concentrated (10X) monoclonal cell culture supernatants 1-13 or IgM standard (see key) were pipetted into the outer wells.

(b) The central well of each rosette contains goat serum against mouse γ_{2B} chain (10μl). Ten μl aliquots of 10X concentrated monoclonal cell culture supernatants 1-13 or IgG_{2B} standard (see key) were pipetted into the outer wells.
After incubation for 48 hours at 4°C all slides were processed and stained for protein as described in section 3.2.15.

Figure 3.2 Ouchterlony Analysis: SLPC vs. Monoclonal Antibodies

The central well of each rosette contains sheep liver pyruvate carboxylase (10μl of enzyme, specific activity 25 units/mg and 2.3mgs/ml). Ten μl aliquots of 10X concentrated monoclonal cell culture supernatants 1-13 (see Key) were pipetted into the outer wells.

After incubation at 4°C for 48 hours the slides were processed and stained for protein as described in section 3.2.15.

Antibody	Key
1.1D4/B9/H11	1
1.1B2/G8/G9	2
2D5/C7/D5	3
1.1D5/A8/D3	4
1.1B2/G8/G6	5
1.1D4/B9/H4	6
2D5/C7/D6	7
2D5/C7/E10	8
1.1D4/B8/C5	9
1.1D4/B9/H3	10
1.1D4/B9/H4	11
1.1D5/A8/E6	12
1.1D5/A8/E8	13
STANDARD IgM	SD
STANDARD IgG_{2B}	SD

FIGURE 3.1

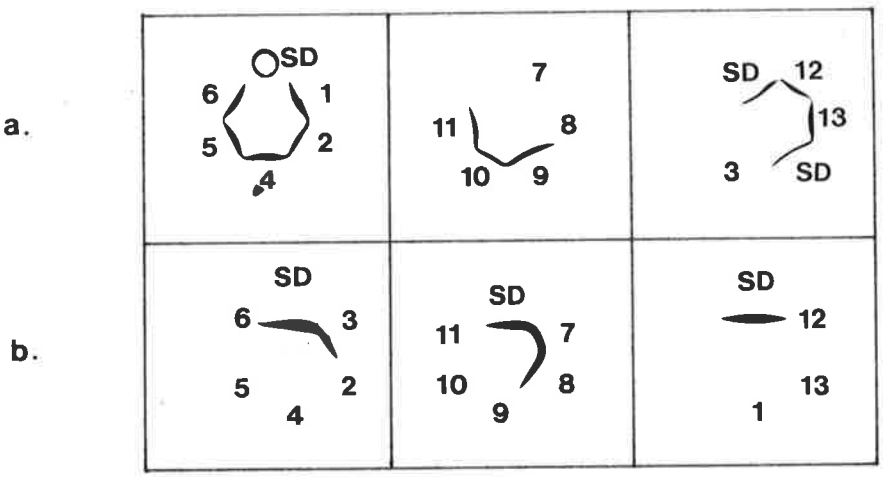


FIGURE 3.2

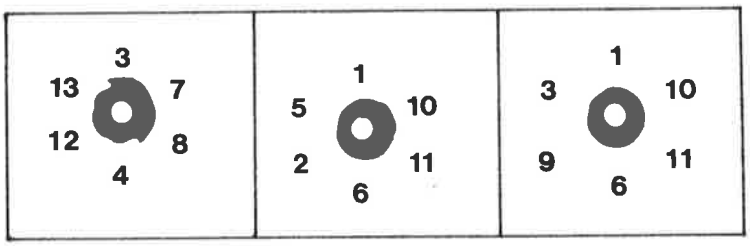


Figure 3.3 SDS Polyacrylamide Gel Electrophoresis of Biotin Enzymes used to Screen Hybridoma Cells

(a) SLPC prepared as described in section 2.2.6 was further purified using Avidin-Sepharose chromatography [see section 2.2.7(b)] and polyacrylamide gel electrophoresis (see section 2.2.14). The purified and partially purified enzyme was electrophoresed on a 15% SDS polyacrylamide reducing gel as described in section 2.2.9.

Lane 1: Active SLPC purified by Avidin-Sepharose chromatography.

Lane 2: Denatured SLPC purified from a polyacrylamide gel.

Lane 3: SLPC (M_r = 117000) before purification by Avidin-Sepharose or gel electrophoresis. The major contaminating band in this preparation is glutamate dehydrogenase (M_r = 55000).

(b) Different pyruvate carboxylases (50 μg) prepared as described in section 2.2.6 and sheep liver propionyl-CoA carboxylase (50 μg) were electrophoresed on a 10% SDS polyacrylamide reducing gel (see section 2.2.9) Lane 1 contains sheep liver propionyl-CoA carboxylase. Liver pyruvate carboxylases from kangaroo (Lane 2), rat (Lane 3), sheep (Lane 4), duck* (Lane 5), pheasant (Lane 6) and chicken (Lane 7) are shown.

* Duck liver pyruvate carboxylase was not used in experiments described in chapters 3 and 4.

FIGURE 3.3 a

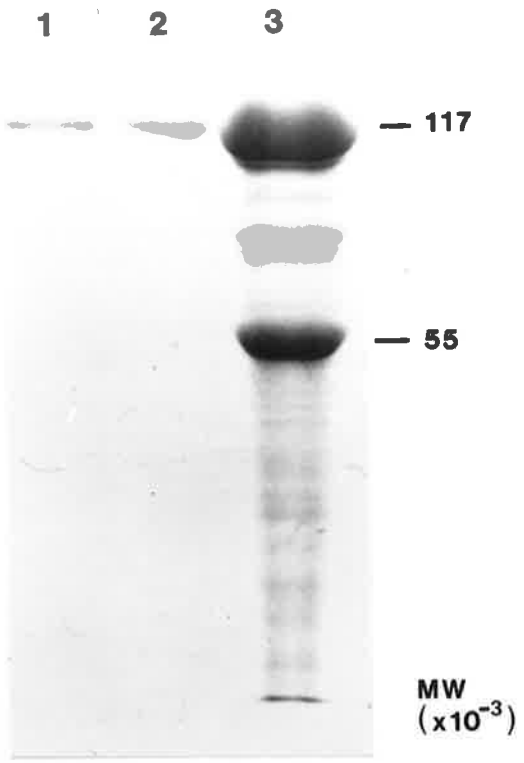


FIGURE 3.3 b

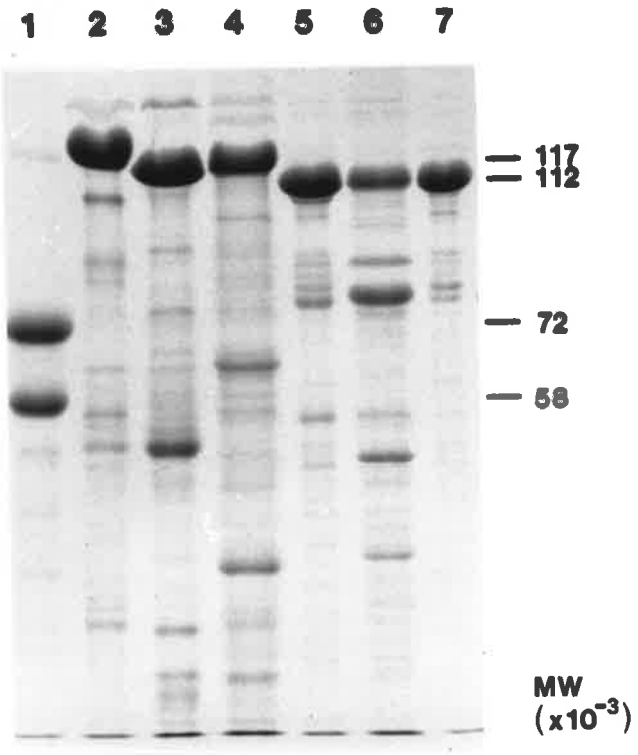


Figure 3.4 Binding of Expanded Samples to Biotin Enzymes

The binding of 44 expanded cell samples to pyruvate carboxylase from the livers of chicken (△), pheasant (◆), rat (x), sheep (◇) and kangaroo (■) and sheep liver propionyl-CoA carboxylase (□) was determined by ELISA as described in section 2.2.10(b). The average absorbance (n=2) at 450nm for each sample on each biotin enzyme is plotted. Both the mean absorbance (X) at 450nm of the negative controls (n=6) and the absorbance two standard deviations above the mean (X + 2s) of the negative control are indicated by the horizontal lines.

KEY	SAMPLE	KEY	SAMPLE
1	C.22.15.1B4	23	C.24.15.2A4
2	3B5	24	2B1
3	3D1	25	2B2
4	3D2	26	2D3
5	4C3	27	2D4
6	4C4	28	3C6
7	C.23.15.1A1	29	3D3
8	1C5	30	3D5
9	1C6	31	4A1
10	1D1	32	4D6
11	2A6	33	C.25.15.1D2
12	2D6	34	2B3
13	3A2	35	2B5
14	3B1	36	2D1
15	3B2	37	4A1
16	3C4	38	4A2
17	4A6	39	4A3
18	C.24.15.1B6	40	4A5
19	1C1	41	4B2
20	1D1	42	4B3
21	1D5	43	4B5
22	1D6	44	4C4

Figure 3.4

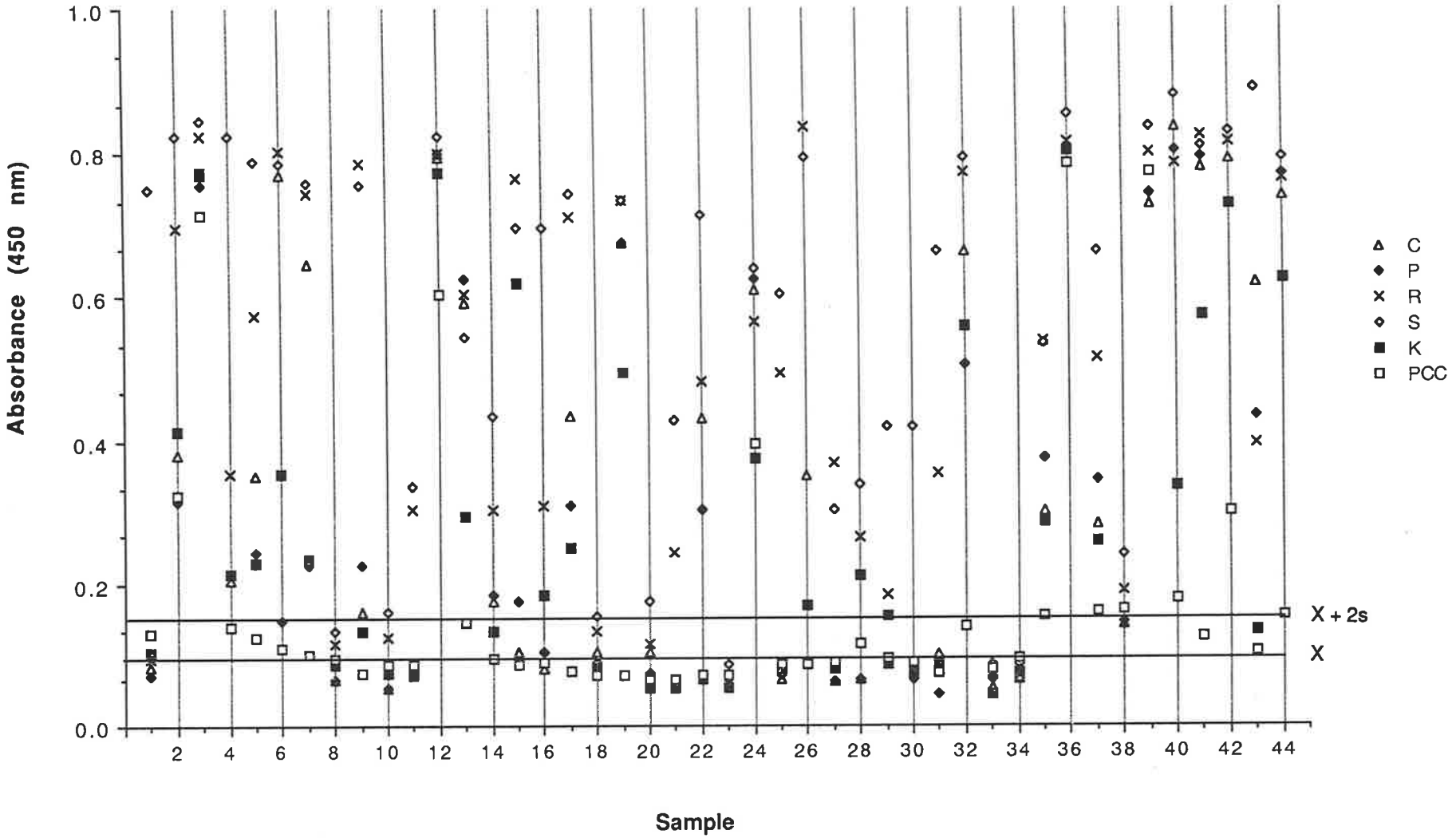
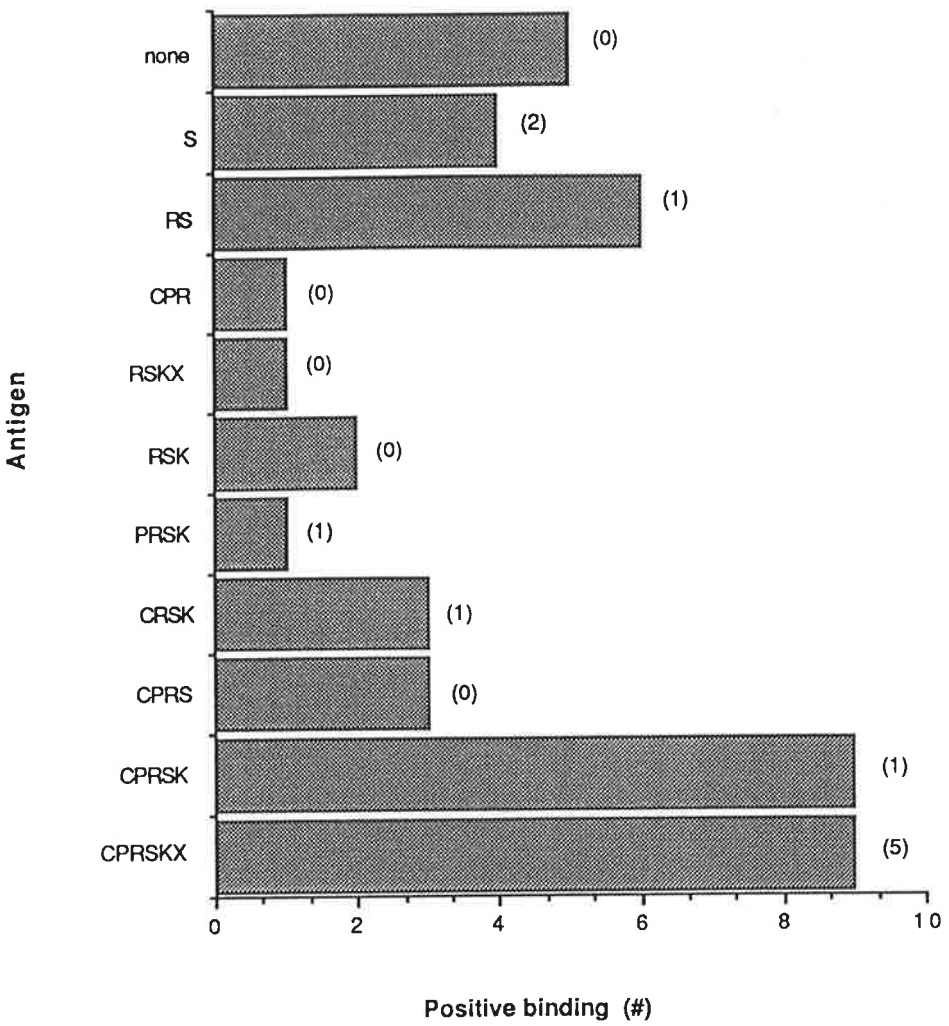

Absorbance (450 nm)
1.0
0.8
0.6
0.4
0.2
0.0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
42
44
Sample
C
P
R
S
K
PCC
X + 2s
X

Figure 3.5



This diagram displays the number of expanded cell samples which are able to bind to different sets of biotin enzymes as determined by the ELISA results shown in Figure 3.4. In brackets, the number of samples in each set that are able to inhibit the activity of SLPC is indicated.

Pyruvate carboxylase from the livers of chicken (C), pheasant (P), rat (R), sheep (S) and kangaroo(K) and propionyl-CoA carboxylase from sheep liver (X) were the antigens studied.

Figure 3.6 Protein A-Sepharose Chromatography of Ascites Fluids Containing Monoclonal Antibodies

Ascites fluid (400 μl) containing monoclonal antibodies 6, 12 , 18, 42, 60, 78, 80, 98 and 113 and 200 μl of 0.1 M sodium phosphate adjusted to pH 8.1 were loaded onto a Protein A-Sepharose CL-4B column (5ml). The column was washed with 30 ml of 0.1 M sodium phosphate pH 8.0. IgG_1 antibodies were eluted with 30 ml of 0.1 M citrate pH 6.0. IgG_{2a} and IgG_{2b} antibodies were eluted with 30 ml of 0.1 M citrate buffers of pH 4.5 and 3.0 respectively. Antibody eluting at pH 4.5 or 3.0 was neutralised immediately with 1M Tris to prevent acid denaturation. The chromatography of antibodies 6, 12, 18, 42 and 80 (a) and 60, 78, 98 and 113 (b) is depicted in Figure 3.6. The column was monitored at a wavelength of 280nm.

FIGURE 3.6 a

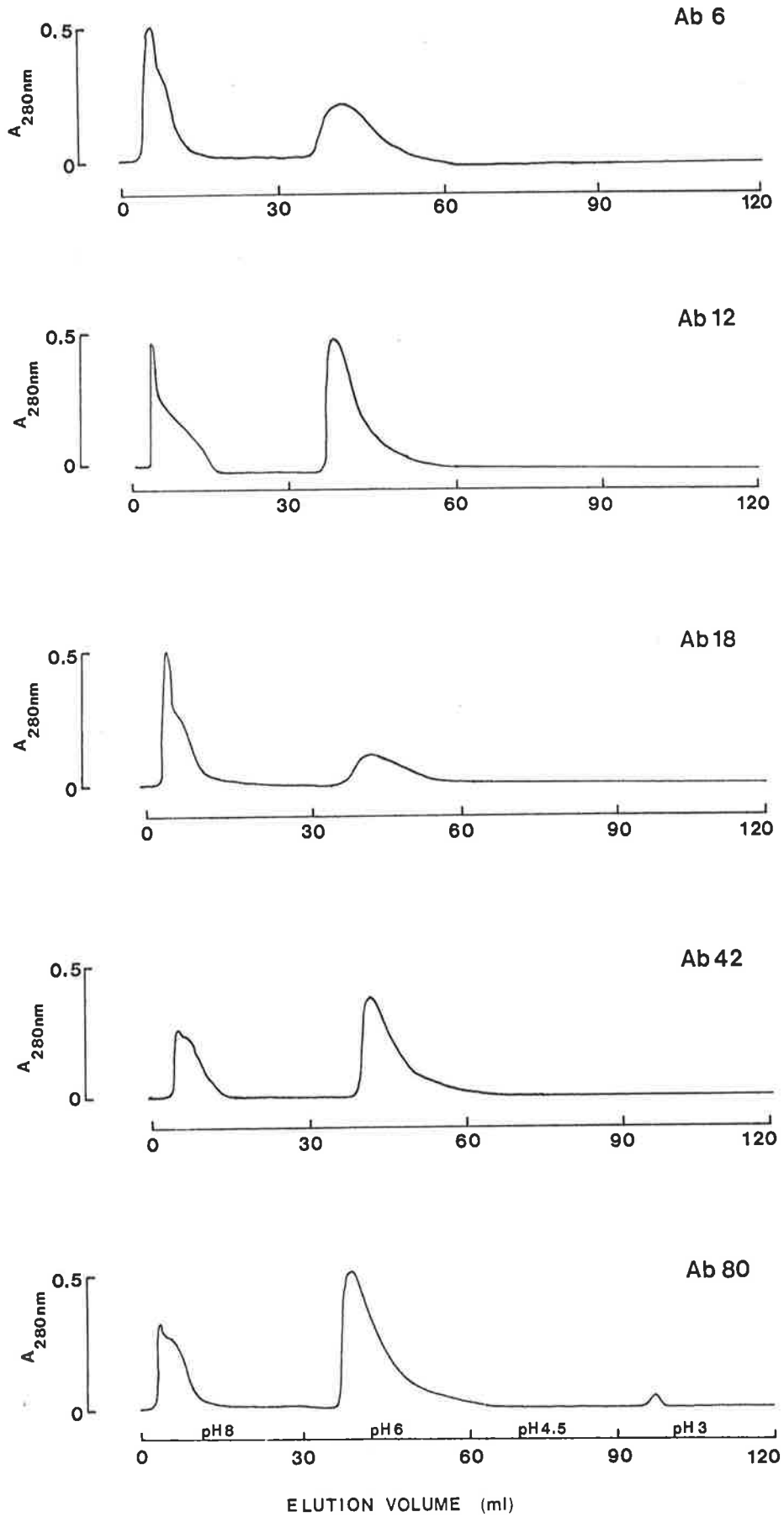
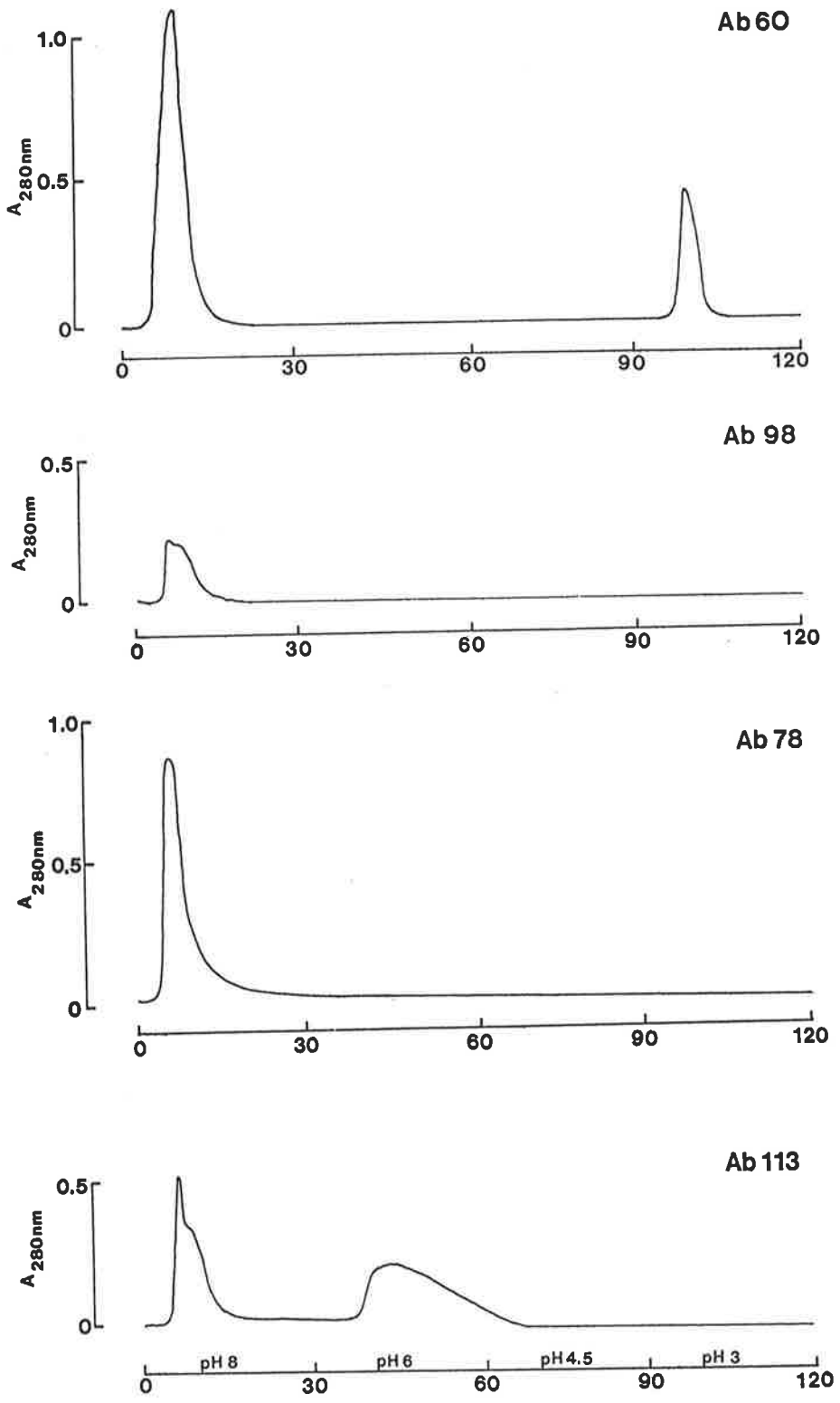

Ab 6
Ab 12
Ab 18
Ab 42
Ab 80
0.5
0
A280nm
0
30
60
90
120
pH8
pH6
pH4.5
pH3
ELUTION VOLUME (ml)

FIGURE 3.6b


Ab60
1.0
0.5
0
A_{280nm}
0
30
60
90
120
Ab 98
0.5
0
A_{280nm}
0
30
60
90
120
Ab 78
1.0
0.5
0
A_{280nm}
0
30
60
90
120
Ab 113
0.5
0
A_{280nm}
pH 8
pH 6
pH 4.5
pH 3
0
30
60
90
120
ELUTION VOLUME (ml)

CHAPTER 4

INITIAL CHARACTERISATION OF MONOCLONAL ANTIBODIES

4.1 INTRODUCTION

The work in this chapter describes the partial characterisation of the monoclonal antibodies whose production was described in Chapter 3. Initial experiments showed that only antibodies from Set B were able to inhibit the activity of SLPC. Since it was considered that the inhibitory antibodies would give greater insight into the relationship between the structure and function of pyruvate carboxylase, only Set B antibodies were characterised further. These included antibodies 6, 12, 18, 42, 60, 78, 80, 98 and 113.

The main aim of this work was to identify the binding sites of the antibodies relative to already identified sites on pyruvate carboxylase. For example, it was hoped that by investigating the nature of inhibition of enzyme activity by the antibodies, that their binding position relative to the active site or subsites could be established. Experiments were conducted to ascertain whether inhibitors of pyruvate carboxylase were directed:

(i) at the biotin moiety;

(ii) close to the first or second subsite of the catalytic centre; or

(iii) close to the acetyl-CoA binding site.

In addition, it was established, with the aid of ELISA based assays:

(i) whether any of the antibodies bound within the region on the enzyme's external face, occupied by the biotin binding protein avidin or antibodies directed towards the biotin moiety;

(ii) whether any of the antibodies bound in the vicinity of the two hyper-reactive lysine residues known to be modified so readily by trinitrobenzenesulphonic acid; and

(iii) in quantitative terms, to which other biotin enzymes besides SLPC these antibodies bound, thus giving some insight into the similarity in structure of the antigenic determinants of these enzymes which share an analogous two step reaction mediated by the biotin moiety.

4.2 RESULTS AND DISCUSSION

4.2.1 The Effect of Monoclonal Antibodies on the Activity of Sheep and Chicken Liver Pyruvate Carboxylase

4.2.1.1 Acetyl-CoA Dependent Assays

Assuming that a 1:1 molar ratio of enzyme subunit to antibody is required to cause inactivation of enzyme activity, then 2 mUnits of SLPC of specific activity 40 units/mg and 2 mUnits of CLPC of specific activity 29 units/mg would be inactivated by 64 and 92ng of IgG antibody respectively. This assumption is based on the molecular masses of the subunits of SLPC and CLPC being 117 kDa and 112 kDa respectively (I. Cassady - personal communication) and antibody of the IgG subclass having a molecular mass of 150 kDa. However, it has been shown by Johannssen et al. (1983) that biotin and therefore the active site is located close to the intersubunit junction on the tetramer of CLPC (see section 1.4.2). Hence it is likely that because the active sites of the enzyme subunits are so close together that only one or two active site directed antibody molecules would be able to bind per tetramer. In addition, the dissociation constant for the antibody and enzyme will have considerable bearing on the molar ratio of enzyme to antibody required to cause inactivation of enzyme activity.

Sheep liver pyruvate carboxylase (40 units/mg; 1.3-2.0 mUnits) and chicken liver pyruvate carboxylase (29 units/mg; 1.5-1.9 mUnits) were incubated with increasing amounts of the monoclonal antibodies 6, 12, 18, 42, 60, 78, 80, 98 and 113, for one hour at 30°C in 75 µl. These antibodies had been isolated from ascites fluid by a 50% ammonium sulphate precipitation and then dialysed against 0.1M Tris-Cl pH 7.2 with 0.1% sodium azide. Acetyl-CoA (200µM) was present during incubation in order to prevent inactivation of the carboxylases by dilution effects. After incubation the activity of the enzymes was measured by the acetyl-CoA dependent radiochemical assay (see section 2.2.1(b)).

Figures 4.1 and 4.2 show the typical effect of antibodies 6, 12, 60 and 78 on the activity of sheep and chicken liver pyruvate carboxylase. Clearly antibodies 6 and 12 show nearly complete inactivation of the sheep liver enzyme with the lowest amount of antibody used. Inactivation by lesser amounts of antibodies 6 and 12 is shown in Figure 4.5. Antibody 12 was also shown to inhibit CLPC activity and subsequent assays (not presented) showed that 2µg of antibody 12 could completely inhibit the activity of the same amount of CLPC and that 0.5µg of antibody showed 80% inhibition. Antibody 6, however, did not inhibit CLPC as it had for SLPC but behaved like the non-inhibitors 60 and 78. The increase of SLPC enzyme activity for antibodies 60, 78 and for control ascites antibodies was unexpected but consistently observed. Antibodies 6, 60 and 78 also activated CLPC activity. One possible explanation for this effect could be that non-specific protein binding sites on the plastic tube were occupied by antibody rather than enzyme molecules and hence this left more active enzyme in solution. This phenomenom was not investigated further and antibodies which behaved in this manner or had no effect on enzyme activity were termed "non-inhibitors".

Figures 4.3 and 4.4 show the typical effect of antibodies 113, 18, 42, 80 and 98 on the activity of CLPC and SLPC. Antibodies 113, 18 and 42

were shown to inactivate SLPC activity with antibody 113 being the most potent inhibitor. Once again, the control antibody shows a typical activation of activity for both SLPC and CLPC. Hence antibodies 80 and 98 which act similarly to the control antibody are non-inhibitors. For CLPC only antibody 42 was shown to inhibit the activity. Antibodies 113, 18, 98 and 80 are non-inhibitors of CLPC.

For this set of antibodies, much less antibody was used in comparison to the assays for antibodies 6, 12, 60 and 78. However, for the most potent inhibitor of SLPC (antibody 113) at least 3µg of antibody was required for 95% inhibition which is far in excess of that predicted for an antibody which is binding to the active site. This poses the question as to whether those antibodies which inhibit activity are actually binding elsewhere on the enzyme which induces some conformational change that affects the active site of the enzyme or that the binding of antibody to the enzyme is very weak.

In summary, antibodies 6, 12, 113, 42 and 18 are able to inhibit the activity of SLPC while only 12 and 42 are able to inhibit the activity of CLPC.

4.2.1.2 Acetyl-CoA Independent Assays

For the assays described in section 4.3.1.1 acetyl-CoA was always present both during the incubation of the enzyme with the antibody and when the enzyme activity was being assayed. Sheep liver pyruvate carboxylase has been shown to have some enzyme activity in the absence of the allosteric activator acetyl-CoA. This is known as "acetyl-CoA independent activity" and may approach 25% of the acetyl-CoA dependent activity (Ashman *et al.*, 1972). It was of interest to determine whether the antibodies affected the acetyl-CoA independent activity of SLPC. Monoclonal antibodies 6, 12, 60 and 78 were examined. Unfortunately, time

did not permit the acetyl-CoA independent assays to be carried out using antibodies 113, 42, 18, 80 and 98. Sheep liver pyruvate carboxylase was incubated with antibody such that the amount of enzyme in the incubate was not below 4 units/ml (to avoid inactivation by dilution). Therefore acetyl-CoA was not required during incubation of enzyme with antibody. A 20-fold molar excess of antibody over enzyme subunits was used. After incubation the activity of the enzyme was determined by both the acetyl-CoA independent and dependent radiochemical assays. The activity is expressed as a percentage of the activity of SLPC alone in both the acetyl-CoA independent and dependent assays (see Table 4.1).

For SLPC alone the acetyl-CoA independent activity was shown to be 12.5% of the dependent activity. Clearly antibodies 6 and 12 were able to affect the activity of SLPC whether it be acetyl-CoA dependent or independent. This implies, firstly, that the antigenic determinants to which antibodies 6 and 12 bind are always available whether acetyl-CoA is present or absent during the incubation of enzyme with antibody. That is, the presence of acetyl-CoA has not resulted in a change in enzyme conformation which allows the antibody to bind and inhibit activity.

Secondly, because the antibodies are able to inhibit in both types of assays, this implies that the antibodies are binding at or near the active site but not the allosteric site. If the antibodies did bind to the acetyl-CoA binding site, then inhibition of enzyme activity would only be seen in the acetyl-CoA dependent assay where acetyl-CoA is necessary for activity. Antibodies 60 and 78 do not inhibit activity in either assay. It was possible that acetyl-CoA in the dependent assays may have prevented antibodies 60 and 78 binding, hence no enzyme inhibition was observed. However if this were the case, inhibition of enzyme activity would then be observed in the independent assays where acetyl-CoA is not present.

In summary, it appears that antibodies 6, 12, 60 and 78 bind to SLPC in the presence or absence of acetyl-CoA, they do not bind at or near the acetyl-CoA binding site and that the binding of acetyl-CoA to SLPC does not induce conformational changes in the enzyme which allows binding of antibodies 6 and 12 to inhibit activity.

4.2.1.3 Effect of Biotin on the Inhibition of Enzyme Activity by Monoclonal Antibodies

In section 3.3.2.4 preliminary analysis of hybridoma cell culture supernatants before cloning out, showed that none of them contained antibodies against the biotin moiety. However, since the properties of hybridoma cells are unstable until cloned out it was necessary to determine if any of the monoclonal antibodies that were able to inhibit the activity of SLPC were directed towards the biotin moiety. Since an essential part of the reaction sequence of all biotin enzymes requires the biotin moiety, anti-biotin antibodies should inhibit all biotin enzymes, unless sterically hindered from doing so. Of those biotin enzymes tested (SLPC and CLPC) with monoclonal antibodies, only antibodies 12 and 42 were able to inhibit both enzymes and therefore are likely candidates for anti-biotin antibodies.

To determine whether the antibodies 12 and 42 are directed against the biotin moiety, increasing amounts of antibodies (0-2μg) were pre-incubated with a vast excess of biotin. Antibodies 6 and 113 are used as controls because they are able to inhibit SLPC activity but not CLPC. Hence it is unlikely that these antibodies are directed towards the biotin moiety to cause inhibition of SLPC. The molar ratio of free biotin to the maximum amount of antibody used in the assays was 1875:1 for antibodies 42 and 113, and 375:1 for antibodies 6 and 12. These antibodies were then incubated with 1-2 mUnits of SLPC as described in

3.3.2.3(b). The amount of antibody used had been shown previously to be able to inhibit SLPC activity by greater than 40%. The activity of the enzyme was then determined radiochemically and compared to the activity of the enzyme incubated with antibody that had not been pre-incubated with biotin. In this system any anti-biotin antibodies should be completely saturated with biotin and hence the activity of SLPC should no longer be inhibited.

From Figures 4.5 and 4.6 it is evident that although a vast excess of free biotin was present, the antibodies were still able to inhibit the activity of SLPC as effectively as the antibodies which were not pre-incubated with biotin. Hence, antibodies 6, 12, 42 and 113 are not directed against the biotin moiety.

4.2.1.4 Effect of Substrates and Products on the Inhibition of Enzyme Activity by Monoclonal Antibodies

The effect of some substrates and products of SLPC on the inhibition of enzyme activity by monoclonal antibodies was studied. Only those antibodies shown to inhibit SLPC activity were tested, ie. antibodies 6, 12, 18, 42 and 113. Substrates tested were pyruvate, Mg^{2+} and $MgATP^{2-}$. The product tested was oxaloacetate.

SLPC (1-3 mUnits) was incubated with an amount of antibody such that the resulting inhibition of enzyme activity by the antibody would be 50% or greater. The product or substrate was incubated simultaneously with SLPC and the antibody. After incubation, the activity of the enzyme was measured radiochemically.

The greatest concentration of the sustrates incubated with antibody and SLPC was equal to the molarity of the substrate used in the enzyme assay. Hence pyruvate was used at concentrations of 1, 2 and 10 mM, Mg^{2+} at a concentration of 7 mM and ATP at a concentration of 2.5 mM.

The greatest concentration of oxaloacetate used was equal to the concentration of pyruvate used in the enzyme assay for SLPC activity (10 mM). Oxaloacetate was also tested at 1 and 2 mM since it was strongly inhibitory in its own right at 10 mM. For each substrate or product, antibodies 6 and 12 were tested in separate experiments from antibodies 18, 42 and 113. Unless otherwise stated, increases or decreases in inhibition were statistically significant as determined by Student's t-test ($p \leqslant 0.05\%$).

a) Oxaloacetate

The effect of oxaloacetate on the inhibition of SLPC activity by monoclonal antibodies is shown in Tables 4.2 and 4.3. When no oxaloacetate was present antibodies 6 and 12 were able to inhibit 58% of SLPC activity (Table 4.2) while antibodies 18, 42 and 113 inhibited 76-89% of SLPC activity (Table 4.3). It was observed that with increasing amounts of oxaloacetate incubated with SLPC in the absence of antibody, significant product inhibition occurred (76-78% inhibition in the presence of 10 mM oxaloacetate). The percentage inhibition by each antibody at each concentration of oxaloacetate was derived from the value where no antibody was present at that particular concentration of oxaloacetate (zero inhibition).

Clearly, even 1 mM oxaloacetate had a dramatic effect on enzyme inhibition by monoclonal antibodies 6 and 12. For antibody 12 there was a large decrease in enzyme inhibition, whereas for antibody 6 the presence of oxaloacetate actually facilitated greater inhibition of enzyme activity. A further increase in the molarity of oxaloacetate did not produce any more change in the inhibition by antibody 6. However, at 10 mM oxaloacetate, antibody 12 no longer inhibited the activity of SLPC. The results presented are typical of several experiments.

As can be seen in Table 4.3, like antibody 6, oxaloacetate facilitated a greater inhibition of enzyme activity for antibody 18. This was also true to a lesser extent for antibody 113. Conversely, oxaloacetate not only protected the enzyme against inhibition of antibody 42, but activation of SLPC was observed as well. This enhancement of activity was greatest at 1 mM oxaloacetate.

b) Pyruvate

The effect of pyruvate on the inhibition of SLPC activity by monoclonal antibodies is shown in Tables 4.4 and 4.5. When no pyruvate was present the amount of antibody used was able to inhibit activity from 66-86%. Unlike the oxaloacetate assays, when no antibody was present increasing amounts of pyruvate did not have a dramatic effect on the activity of SLPC. Where enzyme, pyruvate and antibody were present a small but significant increase in inhibition was observed for all antibodies except antibody 18. This effect was certainly not as dramatic as that caused by oxaloacetate.

c) Mg^{2+}, $MgATP^{2-}$

The effect of Mg^{2+} and $MgATP^{2-}$ on the inhibition of SLPC activity by monoclonal antibodies is shown in Tables 4.6 and 4.7. In the absence of Mg^{2+} or $MgATP^{2-}$ all of the antibodies inhibit SLPC activity by 72-86%. In both tables it is evident that incubation of SLPC with $MgATP^{2-}$ causes a 16-26% drop in enzyme activity. The effect of Mg^{2+} on enzyme alone is less significant. Again the percentage inhibition values are expressed as a percentage of the value where no antibody was present for either Mg^{2+} or $MgATP^{2-}$.

The only outstanding effect of these substrates on antibody inhibition was seen with antibody 42 when incubated with $MgATP^{2-}$. Here a complete reversal of inhibition by this antibody was observed with 10% activation of enzyme activity over the control.

d) Discussion

The protection afforded by some substrates or products against some antibodies can be attributed to one of two possibilities. Firstly, the site to which the substrate/product binds may also be part or all of the binding site of the antibody in question. If this was the case then the vast excess of substrate/product over antibody in these assays would result in decreased binding of the antibody and hence decreased inhibition by the antibody. The second possibility is that the binding of the substrates to the biotin enzyme may cause a conformational change in the enzyme which affects the binding of the antibody to its determinant. It is possible that the antibody may bind either more efficiently or less efficiently in this case.

Attwood et al. (1986) showed that pyruvate, like acetyl-CoA, induces pyruvate carboxylase from chicken and sheep liver to form a 'tight' tetrahedron-like configuration. This configuration enables the enzyme to react with avidin to form avidin-enzyme chains. Hence pyruvate is able to induce conformational changes in the enzyme (although different to the changes effected by oxaloacetate) which may ultimately affect the inhibition of the enzyme by the antibodies.

Incubation of pyruvate carboxylase with oxaloacetate may result in the reversal of the second partial reaction catalysed by pyruvate carboxylase and thus the formation of the carboxybiotin complex. By forming this complex, the conformation of the determinant to which the antibody binds may be altered. Alternatively, if the binding of antibody

causes inhibition of enzyme activity by inducing a conformational change in the enzyme, it is possible that the formation of a carboxybiotin complex may prevent this induction and thus no inhibition of enzyme activity would be observed.

It is known that the magnesium chelate of ATP binds to pyruvate carboxylase. In the presence of ATP(2.5 mM), Mg^{2+}(5 mM), acetyl-CoA(250μm) and HCO_3^-(2.5 mM), pyruvate carboxylase forms a carboxybiotin complex. Goodall et al. (1981) have proposed that there are two forms or states of the enzyme carboxybiotin complex at equilibrium with each other; one state is proposed to be enzyme with carboxybiotin bound at the first sub-site (State I) while the other state (State II) carboxybiotin is unbound but in the vicinity of the first sub-site. Assuming sufficient endogenous HCO_3^- in the solutions used during incubation of enzyme with antibody and substrate, then a carboxybiotin complex will be formed since $MgATP^{2-}$ and acetyl-CoA are also present in these experiments. A slight excess of Mg^{2+}(2 mM) was present. Goodall et al. (1981), have proposed that Mg^{2+} "holds" the carboxybiotin at the first sub-site whereas α-oxo acids, eg. pyruvate, induce the complex to move to the second subsite. Therefore, it is possible in these experiments, that the presence of oxaloacetate results in the carboxybiotin being close to the second subsite and that for the "$MgATP^{2-}$ experiments" the carboxybiotin is closer to the first subsite.

In the presence of oxaloacetate and thus the carboxybiotin complex, inhibition of enzyme activity by antibody 12 is dramatically decreased. Either the determinant to which antibody 12 binds has been altered by the formation of the carboxybiotin complex in such a way that the antibody can no longer bind or the formation of the carboxybiotin complex prevents antibody 12 inducing a conformational change upon binding, that normally results in enzyme inhibition. In the presence of oxaloacetate and

antibody 42, activation of SLPC activity is observed. This implies that antibody 42 must have the ability to bind to the enzyme in the carboxybiotin form. The way in which antibody 42 is able to activate pyruvate carboxylase and why the activation is highest at the lowest concentration of oxaloacetate concentration tested is unclear. Conversely, the presence of oxaloacetate results in increased inhibition by antibodies 18, 113 and especially antibody 6. The combined conformational effects of the binding of the antibodies and the formation of the carboxybiotin enzyme may cause this increased inhibition. Alternatively, the formation of the carboxybiotin may alter the determinants to which these antibodies bind in such a way that the antibodies bind more efficiently to cause greater inhibition.

Pyruvate had little effect on the inhibition and hence binding of all the antibodies tested. Although pyruvate and oxaloacetate share the same binding site on pyruvate carboxylase, their binding induces different conformational changes on the enzyme. Hence, it is not surprising that oxaloacetate affects the inhibition of enzyme activity by some antibodies, yet pyruvate has little effect on the inhibition by the same antibodies.

With the exception of antibody 42, magnesium or the substrate $MgATP^{2-}$ had little effect on antibody inhibition of SLPC. For antibody 42 a 10% activation of enzyme activity over control was observed when $MgATP^{2-}$ was present. Since Mg^{2+} alone did not have any significant effect on the inhibition by antibody 42, the ATP or the combined effect of Mg^{2+} and ATP was responsible for this observation.

It was interesting that oxaloacetate and $MgATP^{2-}$ had the same type of effect on the inhibition of enzyme activity by antibody 42 whereas for antibody 12 only oxaloacetate altered its inhibition of SLPC. As discussed earlier, a carboxybiotin complex may be formed in the presence of either oxaloacetate or $MgATP^{2-}$ (with HCO_3^-, acetyl-CoA)

where the carboxybiotin group is probably close to the second subsite for the former but close to the first subsite for the latter. This suggests then, that the binding of antibody 12 to the enzyme may be closer to the second subsite. However, no matter whether the carboxybiotin was closer to the first or second subsite, the effect of antibody 42 on enzyme activity was drastically altered. At most it can only be suggested that the position of binding of antibody 42 to SLPC is probably close to the active site.

Like antibody 12, only oxaloacetate drastically affected the inhibition by antibody 6. $MgATP^{2-}$ had little effect. Again this suggests that antibody 6 may bind closer to the second subsite of SLPC than the first.

The effect of substrates on the inhibition by antibodies 18 and 113 was of a much lower magnitude than the other antibodies. The position of binding of these antibodies cannot be predicted.

4.2.2 Enzyme-Linked Immunosorbent Assays (ELISA)

4.2.2.1 Monoclonal Antibodies Versus Biotin Enzymes

Enzyme linked immunosorbent assays (ELISA) were performed where different biotin enzymes coated to microtiter plates were probed with monoclonal antibodies. The biotin carboxylases tested were pyruvate carboxylase from the yeast S.cerevisiae and the livers of sheep and chicken, sheep liver propionyl-CoA carboxylase and transcarboxylase from Propionibacterium shermanii. The monoclonal antibodies used were 6, 12, 18, 42, 60, 80 and 113.

Although the biotin enzymes were used in their native form, it was understood that the process of binding to the microtiter plates was likely to denature the enzymes to some degree. The monoclonal cells,

however, were selected initially on the basis of the ELISA results where SLPC in the native form was bound to the microtiter plate. The following ELISAs give some insight as to the similarity of the antigenic determinants of the different biotin enzymes when immobilised to microtiter plates.

The assays were performed as described in section 2.2.10(b). For each antibody, negative controls were included in the assays where biotin enzyme was not coated to the plate. For these wells the binding of the antibodies to proteins from the blocking solution, (0.3% gelatin in PBS), that is, the background binding was being measured. It was assumed that the proteins in the blocking solution did not contain determinants to which the antibodies would bind. This assumption was supported by the low absorbance observed in the ELISA when the antibodies were used to probe gelatin coated wells.

The same batch of antibodies was used throughout the experiments. These antibodies had been purified from ascites fluid by a 50% ammonium sulphate precipitation followed by Protein-A Sepharose chromatography (see section 2.2.13). Six, tenfold dilutions of each antibody were used in triplicate against each biotin carboxylase and the negative controls. The concentration of each undiluted antibody was 1mg/ml. The difference between the plots of the average absorbance at 450 nm versus antibody dilution for the negative controls and the tests is presented in Figures 4.7 to 4.11. For dilutions of the antibody where the absorbance at 450 nm was outside the limits of the Titertek Multiscan ELISA reader, that is, an absorbance of 2.0 or greater, no points were plotted.

Figure 4.7 shows the binding of the monoclonal antibodies to SLPC. Since the monoclonal antibody producing cells were selected on the basis of their antibodies binding to SLPC, it was expected that the binding

observed would be high even at low antibody concentrations. This was observed for all antibodies except antibody 80 which showed weaker binding.

To make comparisons amongst the antibodies and amongst the different biotin carboxylases, the negative log of the antibody dilution at which the absorbance at 450 nm was 0.5 was determined from Figures 4.7 to 4.12. These are presented in Table 4.8. Since the amount of each antibody used to probe the coated microtiter plates was the same at each dilution and assuming that the various biotin enzymes bound to the microtiter wells in an equivalent manner, then the negative log values for absorbance of 0.5 must give a measure of the relative affinities of the antibodies for the biotin carboxylase in question. The greater the negative log value for an absorbance of 0.5, the greater the affinity of the antibody for the antigen.

The same antibodies were used to probe CLPC bound to microtiter plates (see Figure 4.8). Upon comparison with the SLPC ELISA results (see Figure 4.7) it is clear that the curve of antibody response has moved significantly towards the ordinate for antibodies 6, 113 and 18 which indicates less binding to CLPC in comparison to SLPC. Binding by antibodies 12, 42, 60 and 80 remained virtually unchanged. This is also reflected in Table 4.8, where the negative log values have decreased dramatically for antibodies 6, 113 and 18 but remained similar for antibodies 12, 42, 60 and 80.

Figure 4.9 shows the binding of seven monoclonal antibodies to yeast pyruvate carboxylase (YPC). As observed for the pyruvate carboxylases from sheep and chicken liver, the binding of antibodies 12 and 42 is strong in comparison to the other antibodies. The binding of antibody 6 was extremely weak and barely above background. The remaining antibodies did show significant binding to yeast pyruvate carboxylase but it was very weak.

The biotin enzyme, transcarboxylase from Propionibacterium shermanii (TC) was also tested in this system and the results are presented in Figure 4.10. Antibodies 12 and 42 showed the greatest binding to this enzyme. In comparison to the pyruvate carboxylases however, the binding was much weaker for antibody 42. Weak binding was observed for the other antibodies as well. Table 4.8 reflects these findings.

Of all the antibodies tested antibody 60 showed the greatest binding to the propionyl-CoA carboxylase from sheep liver (see Figure 4.11). This binding was only observed at low dilutions of the antibody. Significant but weak binding was observed for antibody 12. The remaining antibodies gave absorbances which were not significantly greater than background binding.

In summary, it appears that antibody 6 binds strongly only to SLPC. Antibodies 18, 80 and 113 bind to all of the pyruvate carboxylases tested with the greatest binding to SLPC. Similarly, antibodies 12, 42 and 60 bind to all of the pyruvate carboxylases as well as exhibiting binding to transcarboxylase. For antibody 60, the binding to the vertebrate pyruvate carboxylases is greater than binding to yeast pyruvate carboxylase or transcarboxylase. Only antibody 60 exhibited reasonably strong binding to SLPCC.

The pyruvate carboxylases and propionyl-CoA carboxylase share the same first partial reaction. Conversely, transcarboxylase and the pyruvate carboxylases share their second partial reaction. All of the biotin enzymes tested have binding sites for CoA derivatives and all have a common biotin attachment site, distinct from the biotin moiety. If the antibodies are directed at or near any of these sites, then it is not surprising that considerable cross-reaction amongst the biotin carboxylases tested was observed.

In previous experiments antibodies 6, 12, 18, 42 and 113 were shown to inhibit SLPC activity while antibodies 12 and 42 inhibited CLPC activity as well. This is reflected in the ELISA results where the binding of antibodies 18, 6 and 113 to CLPC is much lower than to SLPC (see Table 4.8). This indicates that the same antigenic determinants for these antibodies or a similar structure is still present on CLPC but it is not a crucial area involved in the activity of CLPC as it was for SLPC. Antibodies 12 and 42 are able to bind to all of the pyruvate carboxylases tested as well as transcarboxylase. They inhibit chicken and sheep liver pyruvate carboxylase activity. The effects of these antibodies on the activity of YPC and TC have not been tested. It may be speculated that because the pyruvate carboxylases and transcarboxylase catalyse the same second partial reaction, these antibodies (12 and 42) may bind close to the site of the second partial reaction on these enzymes. It has been observed that the inhibition of SLPC activity by antibodies 12 and 42 is reversed in the presence of oxaloacetate. This suggested at first that the binding of these antibodies was close to the second subsite for enzyme activity. However, a reversal of antibody 42's inhibition was also observed when $MgATP^{2-}$ was present that this antibody's binding may (i) involve both subsites or (ii) be prevented by a conformational change induced in the enzyme by either substrate (oxaloacetate or Mg ATP^{2-}) or (iii) be prevented by the formation of carboxybiotin.

4.2.2.2 ELISA to Determine Location of Antibody Binding Sites Relative to the Avidin Binding Site

(This work was done in collaboration with Dr P.V. Attwood.)

(a) Introduction

Avidin is a tetrameric molecule which binds very specifically and tightly to biotin [$K_d = 10^{-15}$M (Green, 1975)] and to the biotin prosthetic group on pyruvate carboxylase. Using electron microscopy in conjunctin with the protein avidin, the position of the biotin prosthetic group on pyruvate carboxylase was deduced by Johannssen et al. (1983). They showed that when the ratio of avidin to enzyme was between 2:1 and 1:2, linear, unbranched polymers of enzyme-avidin complexes were observed under the electron microscope. At other ratios of avidin to enzyme, single enzyme tetramer-avidin complexes occurred. There appeared to be no gross distortion of the enzyme when polymerised with avidin. Under the electron microscope, avidin appeared to be cuboid in shape. Avidin contains two pairs of biotin binding areas located on opposite sides of the avidin molecule. Each pair is located centrally on the faces of the cuboid with a distance of 2 nm between the biotin binding areas within each pair (Green et al., 1971; Safer et al., 1982). Knowing the dimensions of the enzyme tetramer, avidin, the position of the biotin binding sites on the avidin tetramer and the configuration of the enzyme tetramer and avidin in the unbranched polymers, Johannssen et al. (1983) were able to deduce that the biotin prosthetic group on pyruvate carboxylase was located on the external faces of each subunit probably within 3 nm of the intersubunit junction. Figure 4.12 shows diagrammatic views of complexes between avidin and pyruvate carboxylase.

In the following experiment, five monoclonal antibodies (previously described in Chapter 3) which cross-reacted with chicken liver pyruvate carboxylase in ELISA were used. The monoclonal antibodies were 60, 78, 12, 42 and 83. The first two are non-inhibitors of pyruvate carboxylase activity. Antibodies 12 and 42 have been shown to inhibit both sheep and chicken liver pyruvate carboxylase (see section 4.2.1.1). Antibody 83 inhibits CLPC activity and therefore must bind to this enzyme (results not presented). The binding of antibodies 12, 42 and 60 to CLPC in ELISA was demonstrated in section 4.2.2.1. Antibody 78 in ascites fluid has been shown to bind to CLPC in ELISA (results not shown).

By using an enzyme linked immunosorbent assay (ELISA), the effect of avidin complexed to chicken liver pyruvate carboxylase (CLPC) on the binding of the monoclonal antibodies to the enzyme was investigated. It was assumed that the affinity of avidin for the biotin prosthetic group on the enzyme (K_d = 10^{-10}M) (Duggleby et al., 1982) was greater than the affinity of the antibodies for the enzyme. Using this procedure the position of antibody binding relative to the avidin binding site and thus the active site of the enzyme could be proposed.

(b) Procedure

Linear polymers of avidin and CLPC and single enzyme tetramer/avidin complexes were used to coat microtiter plates. They were prepared by incubating avidin and pyruvate carboxylase in the ratio of 1:1 and 10:1 (biotin binding sites:biotin) respectively. The concentration of avidin was 7.18μM and 71.8μM for the ratios of 1:1 and 10:1 (biotin binding sites:biotin) respectively. Further samples were prepared which also contained avidin and enzyme in the ratios of 1:1 and 10:1, but in which the avidin had been incubated with 2mM free d-biotin for 20 minutes at 30°C before addition of the enzyme CLPC. In

addition, all samples were buffered in 0.1M Tris-Cl pH7.2 and contained 250μM acetyl-CoA. The acetyl-CoA not only preserved the tetrameric structure of the pyruvate carboxylase but also enhanced the formation of the avidin-enzyme complex (Attwood et al., 1986). After the addition of the enzyme to the avidin the mixture was incubated for 2 hours at 30°C.

Each sample of avidin-enzyme complex was diluted in 0.1M Tris-Cl pH7.2 containing 250μM acetyl-CoA such that the CLPC concentration of the solution applied to the plates was 10μg/ml. For the samples where the enzyme to avidin ratio was 1:1, free avidin was added just before application to the microtiter plates so that the concentration of avidin in all diluted samples was the same. This was done so that any non-specific binding of antibody to avidin would be equivalent for all samples. The ELISA was carried out as described in section 2.2.10(b) using the monoclonal antibodies to probe the avidin/CLPC complexes. Antibodies 42 and 83 from cell culture supernatants were used at dilutions of 1:100 and 1:1000 respectively. Ascites fluids containing antibodies 12, 60 and 78 were diluted $1:10^5$ for antibodies 12 and 60 and 1:1000 for antibody 78. The dilutions of ascites fluid or culture supernatant used were such that they gave absorbance values at 450 nm of 0.5 to less than 2.0 when used to probe CLPC only on microtiter plates. A 1:1000 dilution of enzyme linked second antibody (horseradish peroxidase linked to rabbit anti-mouse immunoglobulins) was employed. In these assays the colour developing time with the substrate was 5 minutes instead of 10. This allowed high levels of antibody to be used which saturated the enzyme with antibody, but still gave absorbance values at 450nm in the assay which were within the range of the ELISA reader (ie $\leqslant 2$).

(c) Results and Discussion

Table 4.9 shows the results of a typical experiment where the values in the table are the average absorbance values (n = 3) for the ELISA at 450nm ± the standard deviation about the mean. Comparisons were not made between the "1:1" and "10:1" values for each antibody since these experiments were carried out on different plates and it was suspected that there was some plate variation in this case. Comparisons were made for each antibody within a "1:1" or "10:1 group", between the groups where avidin was pre-incubated in the presence or absence of biotin before incubation with CLPC. Two-tailed t tests were performed on each pair (ie. ± biotin), for each antibody at each avidin:enzyme ratio to determine whether the mean of each group was different at the 5% level of significance. The observed t value for each pairwise comparison is displayed in Table 4.9.

For the ratio of avidin:CLPC of 1:1 significant differences between the means for the "plus" and "minus" biotin groups were observed for all antibodies except 78. For antibodies 60 it is clear that when avidin has not been pre-incubated with biotin there is more binding to CLPC. Pre-incubation of avidin with free biotin results in all biotin binding sites on the avidin being blocked and therefore unavailable for binding with biotin on CLPC. It appears therefore that the binding of avidin facilitates increased binding of this antibody to CLPC. This enhanced binding effect, however, is relatively small compared with the significant exclusion effected by avidin on antibodies 12, 42 and to a lesser extent 83, and may therefore reflect a subtle conformational change (not detectable by electron microscopy) induced in the enzyme by its formation of a complex with avidin. The conformational change may be related to avidin pulling the subunits within each dimer pair closer to each other and thus leaving a bigger clearance on the reverse side of the

dimer between the dimer pairs. The diminished binding of antibodies 12, 42 and 83 in the presence of avidin indicates that these antibodies bind at or close to the site of avidin binding or that the binding of avidin causes a substantial conformational change in CLPC which alters the antigenic sites to which these antibodies bind. Even a small conformational change in the enzyme (not detectable by electron microscopy) would be capable of altering antigenic determinants. Another possibility is that these antibodies may be excluded sterically from binding by the formation of enzyme/avidin chains, ie. anywhere on the external faces of the enzyme subunits which encompass the avidin molecules except for the ends of the subunit. This last possibility is distinguishable by comparing the results obtained for 1:1 avidin:enzyme complexes with those for the 10:1 avidin:enzyme mixtures. Furthermore, pre-incubation of the avidin with biotin will prevent avidin binding to CLPC. If an antibody has been prevented from binding sterically due to chain formation of CLPC and avidin (at ratios of 1:1) and not by avidin itself, then no difference in the binding of the antibody should be observed in the presence and absence of biotin for avidin:CLPC ratios of 10:1. Similarly, if the binding of avidin has facilitated the binding of antibody 60 for avidin : enzyme ratios of 1:1, then this should also be observed at ratios of 10:1.

Looking at the "10:1 results" in Table 4.9, clearly there is a large increase in binding of antibodies 12, 42 and 83 where avidin has been pre-incubated with biotin. Two-tailed t tests showed that their mean values (ie. $\pm$ biotin) were statistically different at the 5% level of significance. Since there is more binding of antibodies 12, 42 and 83 in the presence of biotin for both 1:1 and 10:1 ratios of avidin:CLPC, this strongly suggests that these antibodies bind at or near the site to which avidin binds on the enzyme.

At avidin to enzyme ratios of 10:1 no significant difference was observed in the presence and absence of biotin for antibody 78. This was also observed for ratios of 1:1 of avidin to enzyme. Clearly the binding of avidin to the enzyme and the formation of enzyme/avidin polymers has no effect on the binding of this antibody. Therefore, it is proposed that the binding site of antibody 78 is distal to the avidin binding site on the enzyme - perhaps at the ends of the enzyme subunits.

At a 10:1 avidin to enzyme ratio, the binding of avidin to the enzyme has no significant effect on the binding of antibody 60. This is in contrast to the 1:1 ratio where the presence of avidin/enzyme chains allows better binding of the antibody. If avidin bound to the enzyme was inducing a subtle conformational change in the enzyme which did allow the antibody to bind more freely to the internal face of the enzyme dimer (i.e. the face of the dimer which is closest to the other dimer of the tetramer), then surely this would also be observed at 10:1 ratios of avidin : enzyme where two avidin molecules are bound to free tetramers. Again it must be noted that the differences observed in the presence and absence of biotin during pre-incubation were not large in comparison to those observed for antibodies 12, 42 and 83.

Figure 4.12(c) shows diagrammatically, the dimers from two adjacent tetramers in an avidin/enzyme chain, encompassing avidin, and an IgG molecule drawn to scale. For antibodies 60 and 78 which are still able to bind to pyruvate carboxylase in the presence of avidin (whether in chains or as free tetramers), it seems likely that given the dimensions of the antibodies, they would only be able to bind to the ends, the edges or on the internal faces (i.e. the face of the enzyme which has no contact with avidin) of the enzyme subunit.

Avidin binds to the biotin prosthetic group on CLPC which is located in the active site of the enzyme. Excluding the possibility that the binding of avidin causes the alteration of an antigenic determinant

distal to the avidin binding site, it can be proposed, firstly, that those antibodies which were excluded from binding by the presence of avidin on CLPC (both in the chain form and as single enzyme tetramer complexes) bind at or near the active site of the enzyme. Secondly, it follows then that those antibodies which are not excluded by avidin do not bind at or near the active site. Antibodies 12, 42 and 83 fit the former case while antibodies 60 and 78 fit the latter. Antibodies 12, 42 and 83 have also been shown to inhibit CLPC activity. It appears that in this case antibodies which are inhibitors of enzyme activity cannot bind to the enzyme in the presence of avidin. Hence, these antibodies are likely to inhibit by binding at or in close proximity to the active site of the enzyme.

4.2.2.3 Competitive ELISA Using Monoclonal, Anti-Biotin and Anti-TNP Antibodies and Avidin

(The work presented in this section was done in collaboration with Mr Grant Booker)

Two lysyl residues on SLPC are known to be modified with trinitrobenzenesulphonate (TNBS) using the conditions described in section 2.2.12, resulting in the loss of acetyl-CoA dependent activity. If acetyl-CoA is present during modification, one of these lysine residues is protected and there is no effect on catalytic activity (Ashman et al., 1973). It is unresolved whether the modification in the absence of acetyl-CoA occurs at the acetyl-CoA binding site or the modification causes a conformational change in the enzyme which affects the acetyl-CoA binding site.

Using TNBS modified SLPC (TNP-SLPC), the position of these lysine residues was investigated by observing the binding of anti-TNP antibodies in the presence of avidin, anti-biotin antibodies and several monoclonal antibodies. The lysine residue modified in the presence of acetyl-CoA will hitherto be known as Lys-B. The other lysine will be called Lys-A. As for section 4.2.2.2, the use of avidin in this sytem was to detect antibodies which bound at or near the biotin attachment site and thus the active site of the enzyme. For the same reasons, the anti-biotin antibodies were used although the area of exclusion of an anti-biotin antibody would be greater than that of avidin (See Figures 4.13 and 5.9). The area of binding on SLPC of monoclonal antibodies in relation to all of the antibodies tested and avidin was of interest.

The initial experiments involved the titration of each antibody to be used in the competitive ELISA system against SLPC or TNP-SLPC using a normal ELISA system described in section 2.2.10(b). These antibodies were monoclonal antibodies (in their Protein A - purified form) 6, 12, 18, 42, 60 and 80, rabbit serum containing anti-TNP antibodies and goat and rabbit serum containing anti-biotin antibodies. The preparation of the monoclonal antibodies was described in Chapter 3. Anti-biotin antibodies were prepared by immunising rabbits with biotinylated thyroglobulin. Anti-TNP antibodies were a gift from Dr L. Ashman. The lowest dilutions or concentrations at which the final absorbance in the ELISA were 2.0 and 1.0 (ie the dilutions or concentrations required for challenge and test antibody respectively) were determined for each individual antibody on TNBS modified and unmodified SLPC and are listed with Table 4.10(a).

Modification of SLPC with TNBS did not affect the binding of 6, 12, 60 or anti-biotin antibodies. Conversely modification did decrease the binding of antibodies 18, 42 and 80 to the enzyme. However, the difference was relatively small, hence the same antibody concentration

was used for both the modified and unmodified enzyme. In the initial titration, the binding of anti-TNP antibodies to TNP-SLPC modified in the absence of acetyl-CoA was approximately two-fold greater than to SLPC modified in the presence of acetyl-CoA. This was expected if an extra lysine was labelled with TNBS in the absence of acetyl-CoA and if the affinities of the antibodies for the two lysines were equivalent. However, this result was not consistent as is evident in Table 4.10(a) where anti-TNP antibodies are the test antibody and there is no challenge. For the same anti-TNP concentration the binding to SLPC modified in the absence of acetyl-CoA is greater, but not two-fold greater than to SLPC modified in the presence of acetyl-CoA.

The competitive assays were carried out as described in section 2.2.11, and the results of a typical experiment are presented in Tables 4.10(a) and (b). Table 4.10(a) gives the mean absorbance values at 450 nm with the standard deviation about the mean. Determination of these values included the use of control wells for which the contribution of the challenging antibody or serum to the absorbance of the competition assay was ascertained.

Clearly, the absorbance values for the test antibodies binding to SLPC or modified SLPC in the absence of challenging antibodies are less than 1.0 which was the proposed absorbance value for the test antibody alone. In addition to each competition experiment, the binding of the challenge antibody was tested alone in order to confirm that the absorbance value was 2.0 or greater (so that in the competition experiments the binding of the challenge antibody was potentially much greater than the binding of the test antibody and a competitive effect would be observed if the two antibodies bound close to the same site). All challenge antibodies alone gave absorbance values of 2.0 or greater.

The molar ratio of the challenger avidin to SLPC subunits applied to the microtiter wells was 2:1. Because of the high affinity of avidin for SLPC and that only a portion of the SLPC applied to the plate will remain attached, it was assumed that this amount of avidin would compete with an antibody that bound at or near the avidin binding site.

Using the Student's t test for pairwise comparison, it was determined whether the challenge antibody or avidin were able to prevent or decrease the binding of the test antibody to SLPC or TNP-SLPC. To simplify the results, they are expressed in Table 4.10(b) as the binding of the "test" antibody to the antigen in the presence of the challenging antibody or avidin, as a percentage of the binding of the "test" antibody alone. Where the use of a challenge antibody or avidin has resulted in a significant decrease in the binding of the test antibody, this is indicated by * in Table 4.10(b). To validate the system, goat anti-biotin antibodies were "challenged" with rabbit anti-biotin antibodies or with avidin. The "challenging" antibodies and avidin are far in excess of the "test" antibody. Hence it would be expected that these molecules would exclude the binding of the goat anti-biotin antibodies, since they all bind to the same epitope, biotin. This is demonstrated in Table 4.10. Goat anti-biotin antibodies were chosen as the "challenging" anti-biotin antibody in the competition experiments.

The prevention or decrease in the binding of anti-TNP antibodies to TNP modified-Lys-A caused by the challenging antibody or avidin will only be observed when probing SLPC modified in the absence of acetyl-CoA. Conversely, a decrease in the binding of anti-TNP antibodies to TNP modified-Lys-B only, caused by the challenging antibody or avidin, will be observed when probing SLPC modified in the presence or absence of acetyl-CoA. The decrease in binding to TNP-Lys-B on the enzyme modified in the absence of acetyl-CoA will depend on the relative affinities of the TNP antibodies for TNP-Lys-A and B. Assuming that there is

considerable binding of TNP-antibodies to TNP-Lys-A, then it would be expected that a decrease in the binding of anti-TNP antibodies to TNP-Lys-B, caused by a challenging antibody or avidin, would be greater for SLPC modified in the presence of acetyl-CoA (given that the concentration of anti-TNP antibodies employed was the same for SLPC modified in the presence or absence of acetyl-CoA). Finally, if the challenging antibody or avidin causes a decrease in the binding of TNP antibodies to both TNP-Lys-A and B, then the decrease in binding to TNP-SLPC modified in the absence of acetyl-CoA (i.e. where both Lys-A and Lys-B are modified) will be greater than for TNP-SLPC modified in the presence of acetyl-CoA where only Lys-B is modified. The difference between the two will depend on the relative affinities of the TNP-antibodies for the two modified lysine residues.

When goat anti-biotin antibodies or avidin challenged the binding of anti-TNP antibodies to TNP-SLPC, the significant decreases in binding to SLPC modified in the presence or absence of acetyl-CoA were observed (see Table 4.10(b)). This indicates that TNP-Lys-B, at least, is affected since it is the only lysine labelled on SLPC when acetyl-CoA is present. The decrease in binding to the enzyme modified in the absence of acetyl-CoA is less than the enzyme modified in the presence of acetyl-CoA. From the preceding arguments this implies that the binding of anti-TNP antibodies to Lys-B only is decreased in the presence of anti-biotin antibodies or avidin. This suggests that Lys-B lies within the areas of exclusion by the goat anti-biotin antibodies and avidin and thus, close to the active site of the enzyme. This would be unexpected since it is known that modification of Lys-B results in no loss of catalytic activity. Alternatively, the binding of the anti-biotin antibodies or avidin may induce some slight conformational change on the

enzyme subunit resulting in a decrease of binding of the test antibody. Indications of such conformational effects have been reported previously (Choo et al., 1981).

Conversely, when TNP antibodies were used to challenge the anti-biotin antibodies, no significant change in the binding of the anti-biotin antibodies to SLPC modified by TNBS in the presence or absence of acetyl-CoA was observed. This contradiction in results may reflect a higher affinity of the anti-biotin antibodies for the modified enzyme in comparison to the TNP antibodies.

The binding of the inhibitory antibodies 12 and 42 (see section 4.2.1.1) was lowered dramatically in the presence of goat anti-biotin antibodies and avidin confirming the results obtained in section 4.2.2.2. In addition, when antibodies 12 and 42 were used to challenge anti-biotin antibodies, significant decreases were also observed. This suggested that antibodies 12 and 42 may bind close to the active site of these pyruvate carboxylases. The use of anti-TNP antibodies as the challenging antibody caused no significant changes in the binding of antibodies 12 and 42 to TNP-modified SLPC. Conversely, there was a small but significant decrease in the binding of anti-TNP antibodies to SLPC modified in the presence of acetyl-CoA but not to the enzyme modified in the absence of acetyl-CoA when antibody 42 was used to challenge. This suggests that antibody 42 may bind close to Lys-B. Again, this contradition in results may reflect a higher affinity of the monoclonal antibody 42 for the enzyme in comparison to the TNP antibodies.

The binding of monoclonals 6 and 18 to SLPC was unaffected by anti-biotin antibodies or avidin. This was surprising since both of these antibodies inhibit SLPC (but not CLPC activity) (see section 4.2.1.1). Whether antibody 6 was used as a test or challenge antibody, a small but significant decrease in binding of antibody 6 or anti-TNP antibodies to SLPC modified by TNBS in the presence of

acetyl-CoA only was observed, suggesting binding of this antibody close to Lys-B. Only antibody 18 used to challenge anti-TNP antibodies affected the binding to TNP-SLPC modified in the presence of acetyl-CoA.

There was no significant effect of anti-biotin antibodies nor avidin on the binding of antibody 60. Similarly in section 4.2.2.2 avidin had little effect on the binding of antibody 60 to CLPC. Antibody 60 is a non-inhibitor of both CLPC and SLPC activity. A small significant decrease was observed when antibody 60 was used to challenge TNP antibodies on TNP-SLPC modified in the presence of acetyl-CoA suggesting binding near Lys-B. This effect, however, was not dramatic.

Goat anti-biotin antibodies, but not avidin, decrease the binding of antibody 80 to SLPC. Since the area of exclusion by anti-biotin antibodies would be greater than for avidin (See Figures 4.13 and 5.9), this result is not unexpected. The greatest competitive effect with anti-TNP antibodies was observed with antibody 80. When antibody 80 was used either as a test or challenge with TNP antibodies, significant decreases in the binding of test antibody were seen. When antibody 80 was used as the challenge antibody, there is a bigger decrease in binding of TNP antibodies on SLPC modified in the presence of acetyl-CoA. When antibody 80 was used as the test antibody, large decreases in the binding of this antibody were observed on SLPC modified in the presence of acetyl-CoA when TNP-antibodies were challenging. This result can only be due to interaction with TNP-Lys-B. For SLPC modified in the absence of acetyl-CoA, a similar result was obtained which is of approximately the same order as for the enzyme modified in the presence of acetyl-CoA. If TNP-Lys-A was also interacting with antibody 80 (the test antibody), then a compounded effect of both TNP-Lys-A and B on antibody 80 would be observed. This was not seen. Assuming that there is considerable binding of the TNP antibodies to Lys-A when acetyl-CoA is absent (see preceding arguments) it appears that antibody 80 binds close to Lys-B on

the enzyme. Since the anti-biotin antibodies also decrease the binding of antibody 80 to a similar extent, Lys-B and the binding site for antibody 80 may be within the anti-biotin antibody exclusion area, perhaps in the mid-section of the enzyme subunit (since it is known that modification of Lys-B results in no loss of catalytic activity).

To summarise this work it is clear that:

1) Goat anti-biotin, rabbit anti-biotin antibodies and avidin compete for biotin on SLPC thus validating the competitive ELISA system.

2) Both avidin and anti-biotin antibodies compete with antibodies 12 and 42 for binding to SLPC. This implies that these antibodies bind close to the active site of the enzyme. Antibodies 12 and 42 inhibit SLPC and CLPC activity.

3) Conversely, neither avidin nor anti-biotin antibodies affect the binding of antibodies 6, 18 and 60 to SLPC which implies that these antibodies do not bind close to the active site. Antibody 60 is a non-inhibitor of SLPC and CLPC activity. Antibodies 6 and 18 do inhibit SLPC activity but not CLPC activity (see section 4.2.1). Either the amino acids which comprise the binding epitopes for antibodies 6 and 18 are essential for SLPC activity even though they are not within the area of exclusion by avidin, or the binding of the antibodies to these epitopes results in a conformational change to the enzyme which inhibits SLPC activity.

4) Anti-TNP antibodies have the greatest effect on the binding of antibody 80 to TNP-SLPC modified both in the presence and absence of acetyl-CoA. Lys-B is probably close to the binding site for antibody 80. In addition, when both avidin and anti-biotin antibodies were used to challenge anti-TNP antibodies, it was concluded that only binding to Lys-B on TNP-SLPC was decreased, suggesting that Lys-B lies within the areas of exclusion by avidin and biotin antibodies.

5) Anti-biotin antibodies but not avidin compete with antibody 80 for binding to the enzyme. This infers that the site for antibody 80 binding is not near the active centre of the enzyme but within the anti-biotin antibody exclusion area. Antibody 80 is a non-inhibitor of SLPC activity.

From 4 and 5 above it can be concluded that Lys-B and the binding site for antibody 80 are within the anti-biotin antibody exclusion area. However, Lys-B must be closer to the active site than the antibody 80 binding site since avidin does not affect the binding of antibody 80 to SLPC.

To continue this investigation Mr G. Booker has prepared anti-Coenzyme A antibodies by immunising rabbits with CoA-modified albumin. The position of binding of these antibodies to p-azidophenacyl-CoA modified SLPC is to be determined. The p-azidophenacyl-CoA group is an analogue of acetyl-CoA and thus a marker for the acetyl-CoA binding site on SLPC.

4.3 SUMMARY OF CHARACTERISATION OF ANTIBODIES

4.3.1 Interaction with Pyruvate Carboxylase

It has been established that antibodies 6, 12, 18, 42 and 113 inhibit the activity of SLPC. Of these, only antibodies 12 and 42 inhibit CLPC activity. From ELISA experiments only antibodies 12 and 42 bind as efficiently to CLPC as they do to SLPC. Conversely, there is significantly less binding of antibodies 6, 18 and 113 to the chicken enzyme than to the sheep enzyme (see Table 4.8).

How do these antibodies inhibit the activity of pyruvate carboxylase? Since the biotin moiety is common to all biotin enzymes it was of interest to determine whether the inhibitory antibodies were

directed at the biotin moiety. When antibodies 6, 12, 42 and 113 were pre-incubated with vast excesses of free biotin, they were still able to inhibit SLPC activity to the same extent. Hence these antibodies are not directed at this moiety. Antibody 18 was not tested in this system.

Using an ELISA based system, the position of binding of the inhibitory antibodies in relation to the binding of anti-biotin antibodies or avidin on SLPC was investigated. (The binding position of some antibodies relative to avidin on CLPC was also determined.) Clearly, only antibodies 12 and 42 are excluded from binding to SLPC in the presence of anti-biotin antibodies or avidin suggesting that these antibodies (12 and 42) may bind close to the active site of pyruvate carboxylase. Although the presence of pyruvate had little effect on the inhibition of SLPC by these antibodies, oxaloacetate was able to reverse the inhibitory effects of the antibodies and in the case of antibody 42, activation of enzyme activity was observed. In addition $MgATP^{2+}$ was shown to reverse the inhibitory effects of antibody 42 to give activation of enzyme activity. As discussed previously, the presence of oxaloacetate results in carboxybiotin being close to the second subsite whereas the presence of $MgATP^{2+}$ and exogenous HCO_3^- would favour in the carboxybiotin being closer to the first subsite. Since the inhibition by antibody 12 was affected by oxaloacetate only, this antibody may bind close to the second subsite. Because inhibition by antibody 42 is affected by both oxaloacetate and $MgATP^{2+}$, it is difficult to propose a site of binding other than to suggest that it is close to the active site, or that the binding of antibody 42 is incompatible with the formation of carboxybiotin (and vice versa) despite the lack of its interaction with biotin per se.

The inhibitory effect of antibody 6 was enhanced by the presence of oxaloacetate which could imply that the binding site of this antibody is close to the second subsite. However, as antibody 6 was still able to

bind to SLPC in an ELISA in the presence of avidin or anti-biotin antibodies this suggests that the binding site of this antibody is not close to the active site. It is possible, however that antibody 6 may induce a conformational change in the enzyme which alters enzyme activity.

Inhibition by antibodies 18 and 113 was hardly affected by the presence of substrates of the enzyme. In addition, the presence of avidin and anti-biotin antibodies did not prevent antibody 18 binding to SLPC. (Antibody 113 was not tested in this system.) This suggests that these antibodies may bind distant to the active site and exert their effect through a conformational change in the enzyme.

The remaining antibodies 60, 78, 80 and 98 are non-inhibitors of enzyme activity. Antibodies 60 and 80 were not excluded from binding to SLPC and CLPC by the presence of avidin. Similarly, antibody 78 was still able to bind to CLPC when avidin was present. (SLPC not tested.) In addition, antibody 60 was not excluded from binding by anti-biotin antibodies. This implies that antibody 60 must bind at a distance $\geqslant$ 35 nm (the diameter of the antibody combining site - Tzartos et al., 1981) from the active site of the enzyme and that antibody 78 binds at least outside the area of exclusion of avidin. Antibody 80 however was partially excluded from binding when anti-biotin antibodies were present. Since the area of exclusion of anti-biotin antibodies is greater than that of avidin (see Figures 4.13 and 5.9), it is possible that this antibody may bind to the mid-section of the enzyme subunit. In addition, the binding of antibody 80 to TNP-SLPC ($\pm$ acetyl-CoA) was decreased in the presence of anti-TNP antibodies. It was concluded that the site of modification of Lys-B was close to the binding site for antibody 80. This is supported by the observation that anti-biotin antibodies appeared to affect the binding of anti-TNP antibodies to Lys-B only (see section 4.2.2.3). Antibody 98 needs to be characterised more extensively. Table 4.11 summarises all of this work.

4.3.2 Interaction with Other Biotin Carboxylases

In this chapter, the antigenic determinants of different biotin enzymes attached to microtiter plates were studied using monoclonal antibodies prepared against SLPC. This work has been summarised in Table 4.8. Clearly monoclonal antibodies 12, 42 and 60 are able to recognise transcarboxylase from a bacterial source. Although antibody 78 was not included in Table 4.8 it has been shown to bind to SLPCC in ELISA as does antibody 60. Hence, two different biotin enzymes (SLPC and SLPCC) within the same species, from the same organ, share some antigenic determinants. Also of interest is the cross-species comparison of pyruvate carboxylase. There is considerable immunological cross reaction between this enzyme from chicken and sheep liver and yeast.

It appears that the most highly conserved determinants are those to which antibodies 12, 42 and 60 bind. Antibodies 12 and 42 bind close to the active site of the enzyme and are able to inhibit SLPC and CLPC activity. The effect of these antibodies on the activity of TC, YPC and SLPCC would be of great interest. Are these determinants conserved in the enzymes because they are essential for the activity of the enzyme? Conversely antibody 60 is a non-inhibitor of SLPC and CLPC activity. Is this determinant crucial to the correct folding of the enzymes to ensure activity?

Lynen's hypothesis for a family of biotin enzymes arising from the duplication, fusion and divergence of the genes which encode the different functions of the biotin enzyme was discussed in section 1.3.1. Similarity of antigenic determinants amongst biotin enzymes can be related to similarity in structure at these determinants. It is known that an antigenic determinant need only consist of 4-6 amino acids and therefore the question arises as to whether monoclonal antibodies which bind to one antigenic determinant are useful for this type of study on

common structures. Nevertheless, until this time, only polyclonal antibodies have been used for studies of immunological cross reactivity and only in one of these studies was cross reactivity between different biotin enzymes reported (Mottershead et al., 1984). However, in that study Mottershead et al. utilised polyclonal antisera raised against denatured enzymes as immunogens and hence they would have enhanced the proportion of antibodies directed at the more highly conserved regions of these polypeptides normally found in the enzymes' interiors.

The hypothesis of common ancestral genes cannot be accepted on the cross reactivity of the few monoclonal antibodies reported here. However, a large battery of monoclonal antibodies which exhibit immunological cross reactivity amongst different biotin enzymes would give more support to this hypothesis. Ultimately, the best evidence to support or reject this hypothesis will come from a combination of X-ray crystallographic studies and sequencing of the genes coding for the different biotin enzymes. Crystals of sufficient quality and quantity of many biotin enzymes are as yet unavailable. Recently, however, sufficient DNA sequence data from genes encoding several biotin carboxylases has been forthcoming to enable comparisons to be made of the inferred primary structures of these polypeptides (Samols et al., 1988). It is clear that several regions in different enzymes corresponding to specific functional domains share a high degree of homology.

In the following chapter, electron microscopy was used as an additional tool to establish the position of binding of these antibodies on SLPC. Based on all of the results from Chapters 4 and 5 an epitope map of SLPC was produced and is illustrated in Figure 5.9.

Table 4.1 The Effect of Monoclonal Antibodies 6, 12, 60 and 78 on the Acetyl-CoA Dependent and Independent Activity of SLPC

Antibody	% Activity	
	Dependent	Independent
0	100.0	100.0
6	1.4	4.0
12	0	0.1
60	97.6	117.0
78	111.2	106.0

SLPC (0.4 units, 40 units/mg) was incubated with 100μg of monoclonal antibody in 0.1M Tris-Cl pH 7.2 for one hour at 30°C, such that the amount of enzyme in the incubation mix (75 μl) was greater than 4 units/ml. The monoclonal antibodies used were 6, 12, 60 and 78. They had been purified from ascites fluid by a 50% ammonium sulphate precipitation followed by dialysis against 0.1M Tris-Cl pH 7.2, 0.1% sodium azide. The acetyl-CoA dependent and independent activity of SLPC was determined after incubation, for each antibody and when no antibody was present using radiochemical assays as described in section 2.2.1(b) and 2.2.2. The assay time was one minute. The activity of SLPC is expressed as a percentage of the activity of SLPC when no antibody is present for each type of assay. The acetyl-CoA independent activity of SLPC alone was shown to be 12.5% of the dependent activity.

Table 4.2 The Effect of Oxaloacetate on the Inhibition of SLPC Activity by Monoclonal Antibodies 6 and 12

	[Oxaloacetate] mM			
Antibody	0	1	2	10
0	366 ± 11.5 (0)	319 ± 19.5 (0)	235 ± 6.5 (0)	81 ± 3.0 (0)
6	154 ± 27.5 (58)	38 ± 0 (88)	27 ± 5.0 (88)	12 ± 0.5 (85)
12	154 ± 19.0 (58)	288 ± 1.0 (10)	232 ± 5.0 (1)	81 ± 9.5 (0)

SLPC (3.6 mUnits, 23 units/mg) was incubated with 350ng and 600ng of antibodies 6 and 12 respectively in the presence of increasing concentrations of oxaloacetate (0, 1, 2 and 10mM) for 1 hour at 30°C in 0.1M Tris-Cl pH 7.2, 250 μM acetyl-CoA (incubation volume = 75 μl). After incubation the activity of the enzyme was determined radiochemically in a modified acetyl-CoA dependent assay as described in section 3.3.2.3 (b). The values given are mean SLPC activity expressed as mUnits/ml ± the standard deviation about the mean for three replicates.The percentage inhibition by the antibodies at each concentration of oxaloacetate tested (shown in brackets) is derived from the value where no antibody was present for that particular oxaloacetate concentration (ie. zero inhibition).

Table 4.3 The Effect of Oxaloacetate on the Inhibition of SLPC Activity by Monoclonal Antibodies 18, 42, and 113

	[Oxaloacetate] mM			
Antibody	0	1	2	10
0	173 ± 1.0 (0)	118 ± 3.5 (0)	109 ± 3.5 (0)	42 ± 4.0 (0)
18	19 ± 2.0 (89)	10 ± 0.5 (99)	1 ± 0.1 (99)	0 ± 0.2 (100)
42	41 ± 2.5 (76)	219 ± 1.0 (86)*	179 ± 0 (64)*	64 ± 1.5 (52)*
113	19 ± 1.5 (89)	10 ± 0.5 (92)	7 ± 0.1 (94)	2 ± 0.1 (95)

SLPC (1.7 mUnits, 40 units/mg) was incubated with 1.5μg of antibody 113 and 2.8μg of antibodies 18 and 42 in the presence of increasing concentrations of oxaloacetate (0, 1, 2 and 10mM) for 1 hour at 30°C in 0.1 M Tris-Cl pH 7.2, 250 μM acetyl-CoA (incubation volume = 75 μl). After incubation the activity of the enzyme was determined radiochemically in a modified acetyl-CoA dependent assay described in section 3.3.2.3 (b). The values given are mean SLPC activity expressed as mUnits/ml ± the standard deviation about the mean for three replicates. The percentage inhibition by the antibodies at each concentration of oxaloacetate tested (shown in brackets) is derived from the value where no antibody was present for that particular oxaloacetate concentration (ie. zero inhibition). Activation of enzyme activity in the presence of oxaloacetate and antibody is indicated by *.

Table 4.4 The Effect of Pyruvate on the Inhibition of SLPC Activity by Monoclonal Antibodies 6 and 12

	[Pyruvate] mM			
Antibody	0	1	2	10
0	260 ± 12.5 (0)	301 ± 5.0 (0)	286 ± 16.5 (0)	293 ± 6.0 (0)
6	88 ± 16.6 (66)	113 ± 1.5 (64)	97 ± 11.1 (66)	80 ± 19.5 (73)
12	83 ± 6.0 (68)	64 ± 2.0 (79)	58 ± 10.5 (80)	59 ± 12.5 (80)

SLPC (2.6 mUnits, 23 units/mg) was incubated with 350 ng and 600 ng of antibodies 6 and 12 respectively in the presence of increasing concentrations of pyruvate (0, 1, 2 and 10mM) for 1 hour at 30°C in 0.1 M Tris-Cl pH 7.2, 250 μM acetyl-CoA (incubation volume = 75 μl). After incubation the activity of the enzyme was determined radiochemically in a modified acetyl-CoA dependent assay described in section 3.3.2.3 (b). Since pyruvate was present in the samples being assayed, the concentration of pyruvate in the assay mix was adjusted to maintain a concentration of 10mM during the assays. The values given are mean SLPC activity expressed as mUnits/ml ± the standard deviation about the mean for three replicates. The percentage inhibition by the antibodies at each concentration of pyruvate tested (shown in brackets) is derived from the value where no antibody was present for that particular pyruvate concentration (ie. zero inhibition).

Table 4.5 The Effect of Pyruvate on the Inhibition of SLPC Activity by Monoclonal Antibodies 18, 42 and 113

	[Pyruvate] mM			
Antibody	0	1	2	10
0	140 ± 24.5 (0)	154 ± 16.5 (0)	160 ± 5.5 (0)	150 ± 13.0 (0)
18	19 ± 1.5 (86)	18 ± 0.5 (88)	16 ± 1.5 (90)	18 ± 0.5 (88)
42	37 ± 2.5 (74)	28 ± 3.5 (82)	26 ± 0.5 (84)	20 ± 0.5 (87)
113	20 ± 1.0 (86)	19 ± 0 (88)	14 ± 2.0 (91)	11 ± 1.0 (93)

SLPC (1.4 mUnits, 40 units/mg) was incubated with 1.5 μg of antibody 113 and 2.8 μg of antibodies 18 and 42 in the presence of increasing concentrations of pyruvate (0, 1, 2 and 10mM) for 1 hour at 30°C in 0.1 M Tris-Cl pH 7.2, 250 μM acetyl-CoA (incubation volume = 75 μl). After incubation the activity of the enzyme was determined radiochemically in a modified acetyl-CoA dependent assay described in section 3.3.2.3 (b). Since pyruvate was present in the samples being assayed, the concentration of pyruvate in the assay mix was adjusted to maintain a concentration of 10mM during the assays. The values given are mean SLPC activity expressed as mUnits/ml ± the standard deviation about the mean for three replicates. The percentage inhibition by the antibodies at each concentration of pyruvate tested (shown in brackets) is derived from the value where no antibody was present for that particular pyruvate concentration (ie. zero inhibition).

Table 4.6 The Effects of Mg^{2+} and $MgATP^{2-}$ on the Inhibition of SLPC Activity by Monoclonal Antibodies 6, and 12

Antibody	0	Mg^{2+}	$MgATP^{2-}$
0	183 ± 1.5 (0)	203 ± 12.0 (0)	136 ± 7.0 (0)
6	52 ± 6.5 (72)	30 ± 3.0 (85)	20 ± 4.5 (85)
12	50 ± 5.0 (73)	61 ± 1.5 (70)	52 ± 1.5 (62)

SLPC (1.83 mUnits, 23 units/mg) was incubated with 350ng and 600ng of antibodies 6 and 12 respectively in the presence of either Mg^{2+} (7mM) or both Mg^{2+} and ATP (sodium salt) of concentrations 7mM and 2.5mM respectively in 0.1 M Tris-Cl pH 7.2, 250 μM acetyl-CoA (incubation volume = 75 μl). After a one hour incubation at 30°C the activity of the enzyme was determined radiochemically in a modified acetyl-CoA dependent assay described in section 3.3.2.3 (b). Since either Mg^{2+} or $MgATP^{2-}$ was present in the samples being assayed, the concentration of $MgCl_2$ and ATP (sodium salt) in the assay mix was adjusted to maintain concentrations of 7mM $MgCl_2$ and 2.5mM ATP (sodium salt) during the assays. The values given are mean SLPC activity expressed as mUnits/ml ± the standard deviation about the mean for three replicates. The percentage inhibition by the antibodies (shown in brackets) in the presence and absence of Mg^{2+} and $MgATP^{2-}$ is derived from the value where no antibody was present for that particular treatment.

Table 4.7 The Effects of Mg^{2+} and $MgATP^{2-}$ on the Inhibition of SLPC Activity by Monoclonal Antibodies 18, 42 and 113

Antibody	0	Mg^{2+}	$MgATP^{2-}$
0	117 ± 16.0 (0)	115 ± 12.5 (0)	99 ± 5.0 (0)
18	16 ± 0 (86)	14 ± 0 (88)	16 ± 0.5 (84)
42	33 ± 3.0 (72)	30 ± 1.4 (74)	109 ± 1.5 (10)*
113	15 ± 1.0 (86)	12 ± 4.2 (90)	7 ± 1.0 (93)

SLPC (1.17 mUnits, 40 units/mg) was incubated with 1.5μg of antibody 113 and 2.8μg of antibodies 18 and 42 in the presence of either Mg^{2+} (7mM) or both Mg^{2+} and ATP (sodium salt) of concentration 7mM and 2.5mM respectively in 0.1 M Tris-Cl pH 7.2, 250 μM acetyl-CoA (incubation volume = 75 μl). After a one hour incubation at 30°C the activity of the enzyme was determined radiochemically in a modified acetyl-CoA dependent assay described in section 3.3.2.3 (b). Since either Mg^{2+} or $MgATP^{2-}$ was present in the samples being assayed, the concentration of $MgCl_2$ and ATP (sodium salt) in the assay mix was adjusted to maintain concentrations of 7mM $MgCl_2$ and 2.5mM ATP (sodium salt) during the assays. The values given are mean SLPC activity expressed as mUnits/ml ± the standard deviation about the mean for three replicates. The percentage inhibition by the antibodies (shown in brackets) in the presence and absence of Mg^{2+} and $MgATP^{2-}$ is derived from the value where no antibody was present for that particular treatment. Activation of enzyme activity in the presence of Mg^{2+} or $MgATP^{2-}$ and antibody is indicated by *.

Table 4.8 Titre of Monoclonal Antibodies against the Biotin Carboxylases

	Log_{10} Antibody Dilution (A_{450nm} = 0.5)				
Antibody	SLPC	CLPC	YPC	TC	SLPCC
6	4.90	*	*	*	*
12	4.95	5.00	5.30	4.75	*
18	5.20	1.65	1.40	*	*
42	4.85	4.90	5.00	3.85	*
60	5.30	4.70	1.95	1.05	2.70
80	2.30	2.00	1.25	*	*
113	4.30	1.80	1.45	*	*

From Figures 4.7-4.11, the dilution of antibody at which the ELISA absorbance at 450nm was 0.5 was obtained. The negative log value of these dilutions for each antibody for each biotin carboxylase is presented in the table. The concentration of undiluted antibody was 1 mg/ml. Values 1.0 are indicated by *. Antibody 78 has also been shown to bind to SLPC, CLPC and SLPCC by ELISA.

Table 4.9 The Effect of Avidin Complexed to CLPC on the Binding of Monoclonal Antibodies

CLPC was incubated with avidin for 2 hours at 30°C such that the ratio of biotin binding sites on the avidin to biotin on the CLPC was either 1:1 or 10:1 and where the concentration of avidin was either 7.18μM or 71.8μM. As controls, the avidin was incubated with a large excess of free d-biotin (2mM) for 20 minutes at 30°C before addition of CLPC. All samples contained 0.1M Tris-Cl pH 7.2 and 250μM acetyl-CoA.

The ELISA was carried out as described in section 2.2.10(b), except that the development of substrate was for 5 minutes. The CLPC-avidin mixtures (± biotin) were used to coat microtiter plates. Each sample was diluted in 0.1M Tris-Cl pH 7.2, 250μM acetyl-CoA such that the concentration of CLPC was 10μg/ml. For incubates where the avidin:enzyme ratio was 1:1 free avidin was added just before application to microtiter plates so that the concentration of avidin in all diluted samples was the same. This ensured that any non-specific binding of antibody to avidin would be equivalent for all samples. Monoclonal antibodies 12, 60 (diluted 10^5x), 78, 83 (diluted 10^3x) and 42 (diluted 10^2x) were used to probe the avidin/CLPC complexes. Antibodies 12, 60 and 78 were contained in ascites fluid. The remaining antibodies were contained in cell culture supernatants. The values represented in the table are the average absorbance values above zero for the ELISA at 450nm (n = 3) ± the standard deviation. The critical t value at the 5% significance level for four degrees of freedom is 2.776.

+B = pre-incubation of avidin with free biotin.
-B = no pre-incubation of avidin with free biotin.
t = observed t value for each pairwise comparison, +B:-B.

Table 4.9

Antibody	Avidin:CLPC 1:1			Avidin:CLPC 10:1		
	-B	+B	t	-B	+B	t
12	**0.211** ± 0.007	**0.760** ± 0.092	10.306	**0.227** ± 0.016	**0.767** ± 0.117	7.921
42	**0.414** ± 0.016	**1.663** ± 0.125	17.167	**0.300** ± 0.019	**1.411** ± 0.126	15.100
60	**0.665** ± 0.036	**0.515** ± 0.032	5.394	**0.648** ± 0.097	**0.535** ± 0.016	1.991
78	**0.899** ± 0.017	**0.861** ± 0.049	1.269	**0.782** ± 0.143	**0.874** ± 0.045	1.063
83	**0.418** ± 0.007	**0.734** ± 0.003	16.225	**0.346** ± 0.042	**0.679** ± 0.053	7.481

Table 4.10(a) Competitive ELISA to Determine the Position of Binding of Monoclonal, Anti-biotin, Anti-TNP antibodies and Avidin Relative to each other on SLPC and TNP-SLPC

The competitive ELISA was carried out as described in section 2.2.11. The values given are the mean absorbance at 450nm ± the standard deviation about the mean (n = 3). Determination of these values included the use of control wells for which the contribution of the challenging antibody or serum to the absorbance of the competition assay was ascertained.

The binding of the test antibodies in the absence of challenging antibody or avidin to unmodified SLPC [S], SLPC modified by TNBS in the presence of acetyl-CoA [TNP-S (+)] and absence of acetyl-CoA [TNP-S (-)] is shown.

The antibodies or serum used were:

G-Bio = goat anti-biotin serum
R-Bio = rabbit anti-biotin serum
TNP (±) = rabbit anti-TNP serum tested against SLPC which was modified by TNBS in the presence (+) or absence (-) of acetyl-CoA.

Monoclonal antibodies = 6, 12, 18, 42, 60, 80.
Avidin was used as a challenge only.

The following dilutions of serum and concentrations of antibodies and avidin were used:

	Test	Challenge
G-Bio	1:5000	1:100
R-Bio	-	1:5
TNP (±)	1:1000	1:10
6, 12, 18, 60	20ng/ml	1μg/ml
42	100ng/ml	1μg/ml
80	12.7ug/ml	127μg/ml
Avidin	-	11.8μg/ml

Table 4.10 (a)

Challenge Antibody	Test Antibody								
	G-Bio	TNP (+)	TNP (-)	6	12	18	42	60	80
None: S	0.733 ± 0.0.075	--	--	0.465 ± 0.014	0.545 ± 0.026	0.617 ± 0.034	0.387 ± 0.053	0.589 ± 0.031	0.307 ± 0.028
TNP-S (+)	0.530 ± 0.086	0.677 ± 0.015	--	0.540 ± 0.009	0.612 ± 0.068	0.659 ± 0.039	0.490 ± 0.023	0.640 ± 0.012	0.328 ± 0.014
TNP-S (-)	0.448 ± 0.046	--	0.755 ± 0.018	NA	0.562 ± 0.021	0.709 ± 0.024	0.502 ± 0.011	0.621 ± 0.025	0.344 ± 0.008
G-Bio	--	0.542 ± 0.004	0.677 ± 0.012	0.427 ± 0.115	0 ± 0	0.579 ± 0.173	0.126 ± 0.148	0.408 ± 0.141	0.121 ± 0.022
R-Bio	0.028 ± 0.014	nd	nd	nd	nd	nd	nd	nd	nd
Avidin	0 ± 0	0.503 ± 0.014	0.665 ± 0.016	0.455 ± 0.015	0.004 ± 0.112	0.538 ± 0.180	0.011 ± 0.129	0.703 ± 0.029	0.262 ± 0.083
TNP (+)	0.514 ± 0.049	--	--	0.484 ± 0.019	0.526 ± 0.071	0.596 ± 0.028	0.485 ± 0.049	0.691 ± 0.046	0.098 ± 0.036
TNP (-)	0.509 ± 0.016	--	--	NA	0.595 ± 0.099	0.665 ± 0.075	0.445 ± 0.064	0.774 ± 0.066	0.068 ± 0.107
6	0.820 ± 0.033	0.639 ± 0.018	0.711 ± 0.038	--	nd	nd	nd	nd	nd
12	0.548 ± 0.060	NA	NA	nd	--	nd	nd	nd	nd
18	0.628 ± 0.060	0.575 ± 0.011	0.730 ± 0.022	nd	nd	--	nd	nd	nd
42	0.507 ± 0.063	0.571 ± 0.009	0.735 ± 0.018	nd	nd	nd	--	nd	nd
60	0.758 ± 0.055	0.570 ± 0.013	0.739 ± 0.029	nd	nd	nd	nd	--	nd
80	0.672 ± 0.076	0.543 ± 0.020	0.695 ± 0.006	nd	nd	nd	nd	nd	--

NA = not available
nd = not determined

Table 4.10(b) Competitive ELISA to Determine the Binding Position of Monoclonal, Anti-biotin, Anti-TNP Antibodies and Avidin relative to each other on SLPC and TNP-SLPC (% values)

Challenge antibody	Test Antibody								
	G-Bio	TNP(+)	TNP(-)	6	12	18	42	60	80
None	100	100	100	100	100	100	100	100	100
G-Bio	-	80*	90*	92	0*	94	33*	69	39*
R-Bio	4*	nd	nd	nd	nd	nd	nd	nd	nd
Avidin	0*	74*	88*	98	1*	87	3*	119	85
TNP(+)	97	-	-	90*	86	90	99	108	30*
TNP(-)	114	-	-	NA	106	94	89	125*	20*
6	112	94*	94	-	nd	nd	nd	nd	nd
12	75*	NA	NA	nd	-	nd	nd	nd	nd
18	86	85*	97	nd	nd	-	nd	nd	nd
42	69*	84*	97	nd	nd	nd	-	nd	nd
60	103	84*	98	nd	nd	nd	nd	-	nd
80	92	80*	92*	nd	nd	nd	nd	nd	-

The competitive ELISA was carried out as described in section 2.2.11. The values presented in this table are the binding of test antibody to the antigen in the presence of challenging antibody, serum or avidin, as a percentage of the binding of test antibody alone. The actual absorbance values at 450 nm were presented in Table 4.10 (a).

G-Bio = goat anti-biotin serum
R-Bio = rabbit anti-biotin serum
TNP(±) = rabbit anti-TNP serum was tested against SLPC which was modified by TNBS in the presence (+) or absence (-) of acetyl-CoA
Monoclonal antibodies: 6, 12, 18, 42, 60, 80.
NA = not available
nd = not determined
* = competition with challenge antibody causes a significant change in the binding of test antibody (Student's t test, 5% level of significance, n = 3)

Table 4.11 Summary of Characterisation of Monoclonal Antibodies

	Effect on Binding				Effect on Activity					
	α-TNP	α-BIO	Avidin		Inhibition		Prevent Inhibition			
AB	S	S	S	C	S	C	BIO	PYR	OAA	MgATP
6	–	–	–	NT	+	–	–	–	–	–
12	–	+	+	+	+	+	–	–	+	–
18	–	–	–	NT	+	–	NT	–	–	–
42	–	+	+	+	+	+	–	–	+	+
113	NT	NT	NT	NT	+	–	–	–	–	–
60	–	–	–	–	–	–				
78	–	–	NT	–	–	–	INHIBITORS ONLY TESTED			
80	+	+	–	–	–	–				
98	NT	NT	NT	NT	–	–				

KEY

+	effect	α-BIO	anti-biotin antibodies
–	no effect	BIO	biotin
NT	not tested	PYR	pyruvate
AB	monoclonal antibody	OAA	oxaloacetate
α-TNP	anti-TNP antibodies	C,S	chicken, sheep liver pyruvate carboxylase

Figure 4.1 Effect of Monoclonal Antibodies 6, 12, 60 and 78 on SLPC Activity

SLPC (1.3 mUnits; 40 units/mg) was incubated with increasing amounts of monoclonal antibodies 6, 12 60, 78 and control (C)* (0-50μg) in 200μM acetyl-CoA and 0.1M Tris-Cl pH 7.2 (incubation volume = 75 μl) for one hour at 30°C. After incubation, the enzyme activity was measured radiochemically using a modified acetyl-CoA dependent assay as described in section 3.3.2.3(b). The activity of each sample was expressed as a percentage of the activity of the enzyme when no antibody was present. The percent activity is plotted versus the amount of antibody in the incubation for each antibody. Each point represents an average of three experiments.

* control monoclonal antibodies were isolated from cells producing antibody against an antigen unrelated to pyruvate carboxylase.

Figure 4.2 Effect of Monoclonal Antibodies 6, 12, 60 and 78 on CLPC Activity

CLPC (1.5 mUnits; 29 units/mg) was incubated with increasing amounts of monoclonal antibodies 6, 12, 60 and 78 (0-50μg) in 200μM acetyl-CoA and 0.1M Tris-Cl pH 7.2 (incubation volume = 75 μl) for one hour at 30°C. After incubation, the enzyme activity was measured radiochemically using a modified acetyl-CoA dependent assay as described in section 3.3.2.3(b) except that the pH of the assay solution was 7.8. The activity of each sample was expressed as a percentage of the activity of the enzyme when no antibody was present. The percent activity is plotted versus the amount of antibody in the incubate for each antibody. Each point represents an average of three experiments.

FIGURE 4.1

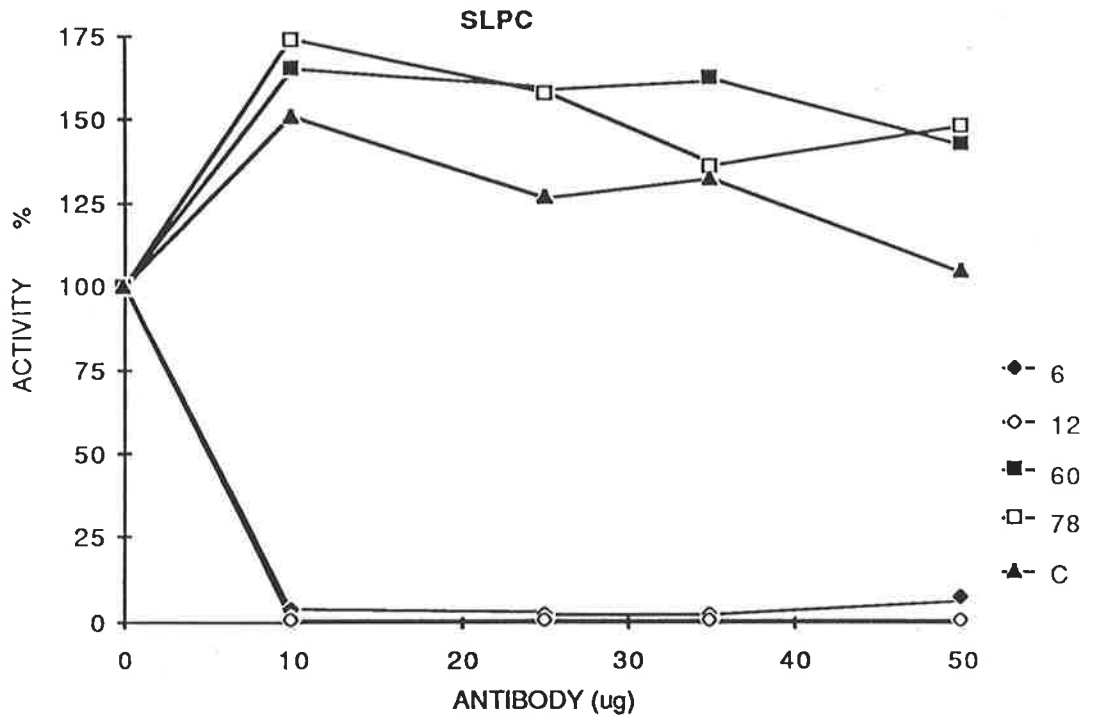


FIGURE 4.2

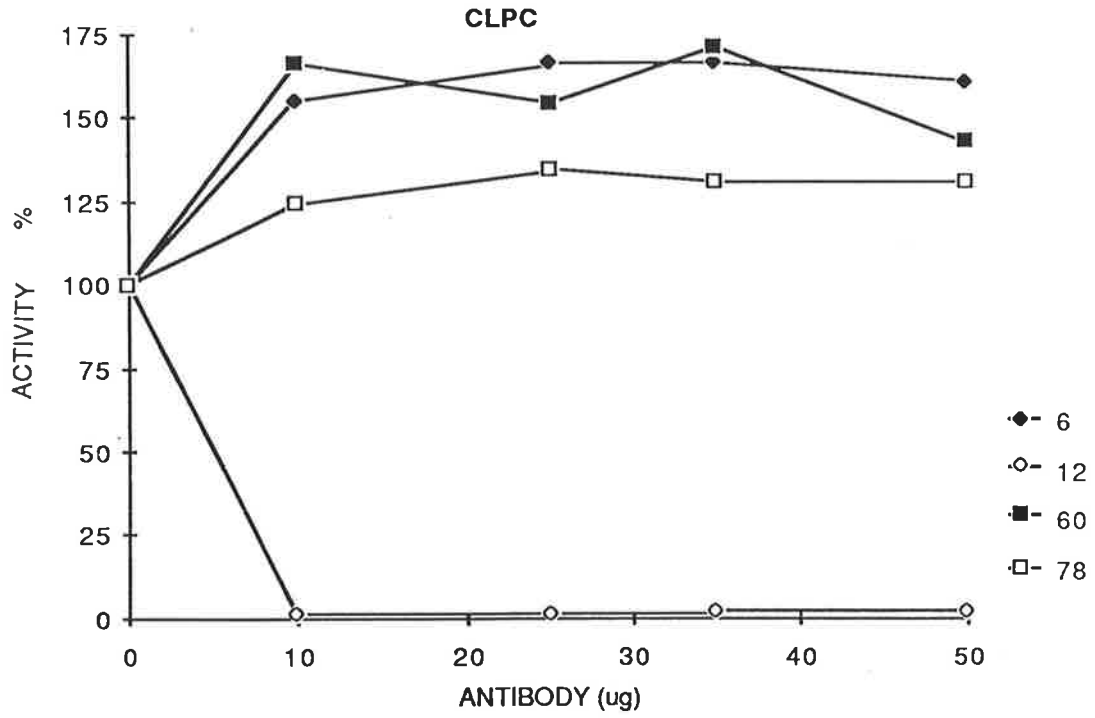


Figure 4.3 Effect of Monoclonal Antibodies 18, 42, 80, 98 and 113 on SLPC Activity

SLPC (2 mUnits; 40 units/mg) was incubated with increasing amounts of monoclonal antibodies 18, 42, 80, 98 and 113 and control (C)* (0-3μg) in 200μM acetyl-CoA and 0.1M Tris-Cl pH 7.2 (incubation volume = 75 μl) for one hour at 30°C. After incubation, the enzyme activity was measured radiochemically using a modified acetyl-CoA dependent assay as described in section 3.3.2.3(b). The activity of each sample was expressed as a percentage of the activity of the enzyme when no antibody was present. The percent activity is plotted versus the amount of antibody in the incubate for each antibody. Each point represents an average of three experiments.

* control monoclonal antibodies were isolated from cells producing antibody against an antigen unrelated to pyruvate carboxylase.

Figure 4.4 Effect of Monoclonal Antibodies 18, 42, 80, 98 and 113 on CLPC Activity

CLPC (1.9 mUnits; 29 units/mg) was incubated with increasing amounts of monoclonal antibodies 18, 42, 80, 98 and 113 and control (C)* (0-3μg) in 200μM acetyl-CoA and 0.1M Tris-Cl pH 7.2 (incubation volume = 75 μl) for one hour at 30°C. After incubation, the enzyme activity was measured radiochemically using a modified acetyl-CoA dependent assay as described in section 3.3.2.3(b) except that the pH of the assay solution as 7.8. The activity of each sample was expressed as a percentage of the activity of the enzyme when no antibody was present. The percent activity is plotted versus the amount of antibody in the incubate for each antibody. Each point represents an average of three experiments.

* control monoclonal antibodies were isolated from cells producing antibody against an antigen unrelated to pyruvate carboxylase.

FIGURE 4.3

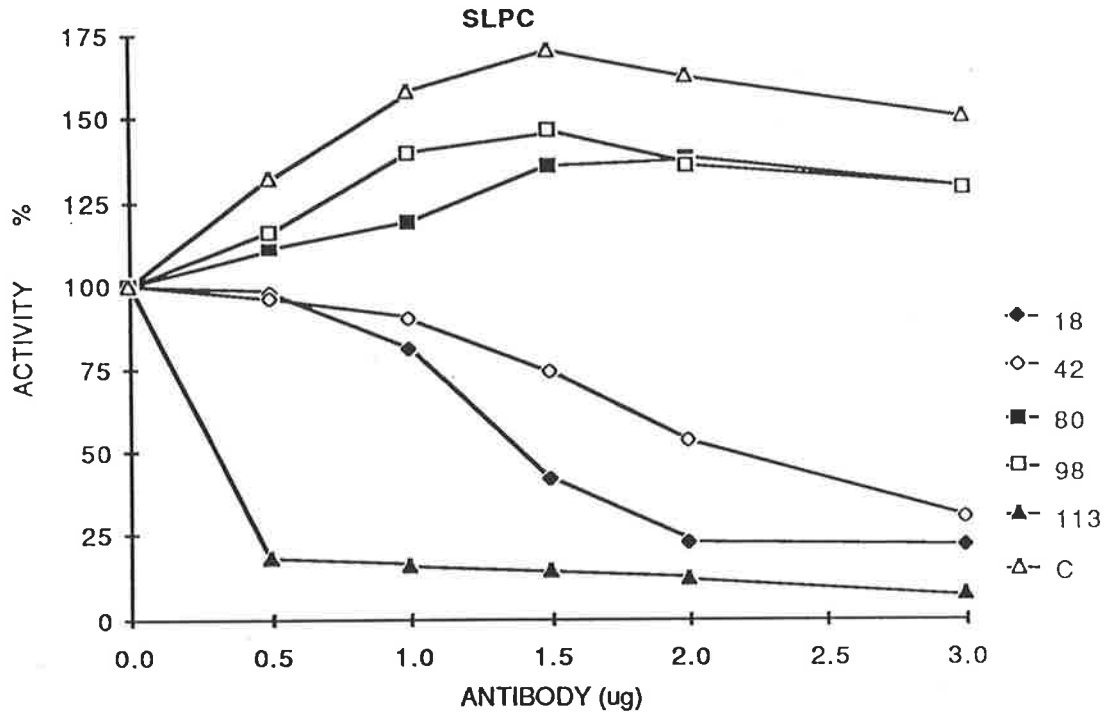


FIGURE 4 4

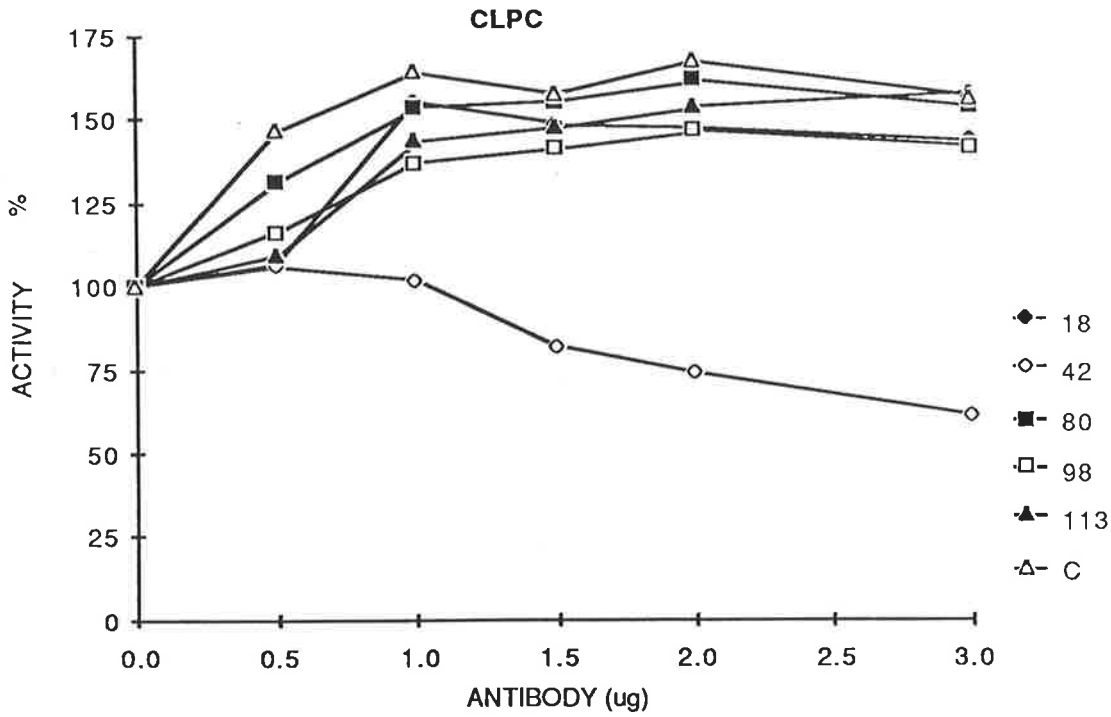
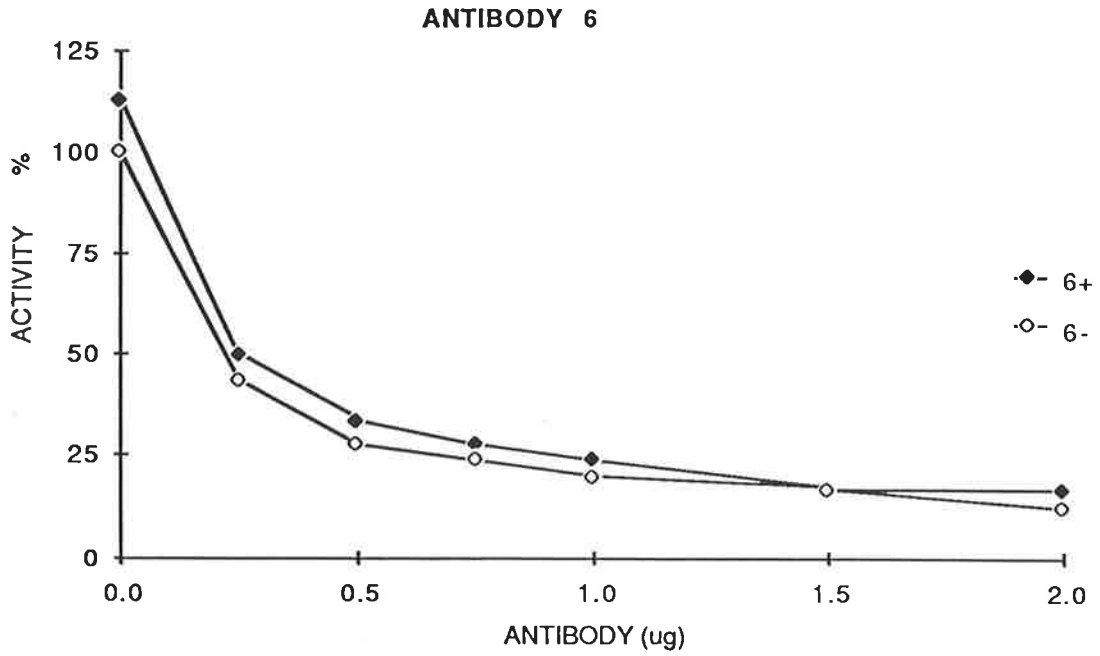


Figure 4.5 Effect of Biotin on the Inhibition of Enzyme Activity by Monoclonal Antibodies 6 and 12

Monoclonal antibodies 6 and 12 (0-2μg) were incubated in 0.1M Tris-Cl pH 7.2 and 200μM acetyl-CoA for 30 minutes at 30°C in the presence and absence of 5 nmoles of biotin (incubation volume = 65 μl). SLPC (1.3 mUnits in 10 μl; 40 units/mg) was added to the antibodies and incubated for a further 60 minutes at 30°C. After this time the enzyme activity was determined radiochemically as described in section 3.3.2.3(b). The activity of the enzyme was expressed as a percentage of the activity of SLPC incubated without antibody or biotin. The percent activity is plotted versus amount of antibody used.

FIGURE 4.5


ANTIBODY 6
125
100
75
50
25
0
ACTIVITY %
0.0
0.5
1.0
1.5
2.0
ANTIBODY (ug)
6+
6-

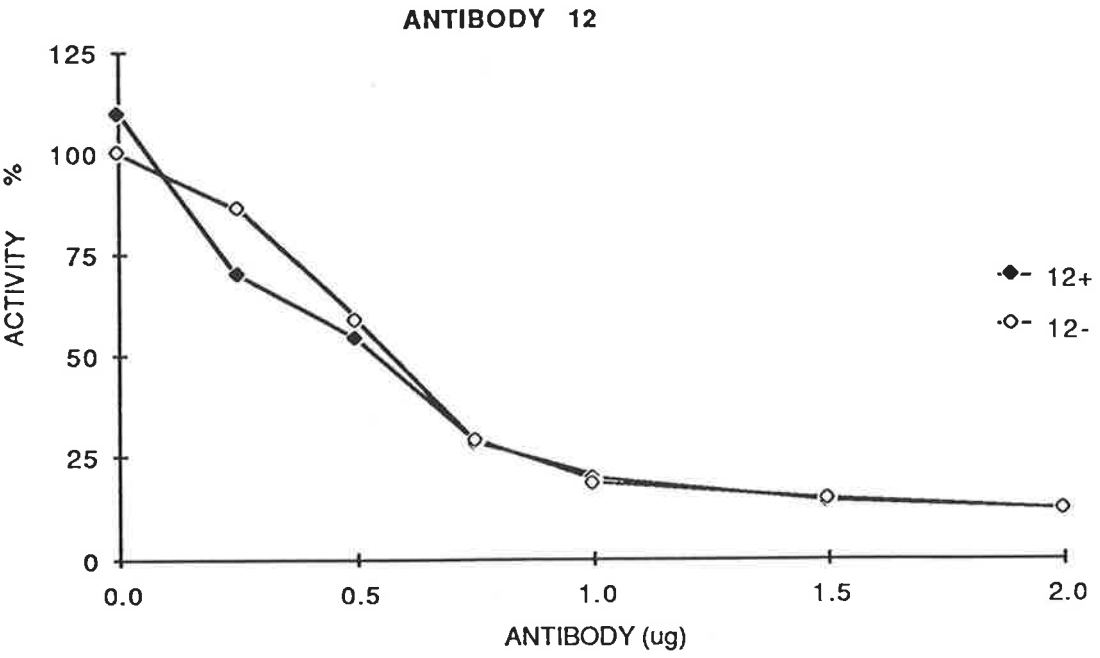
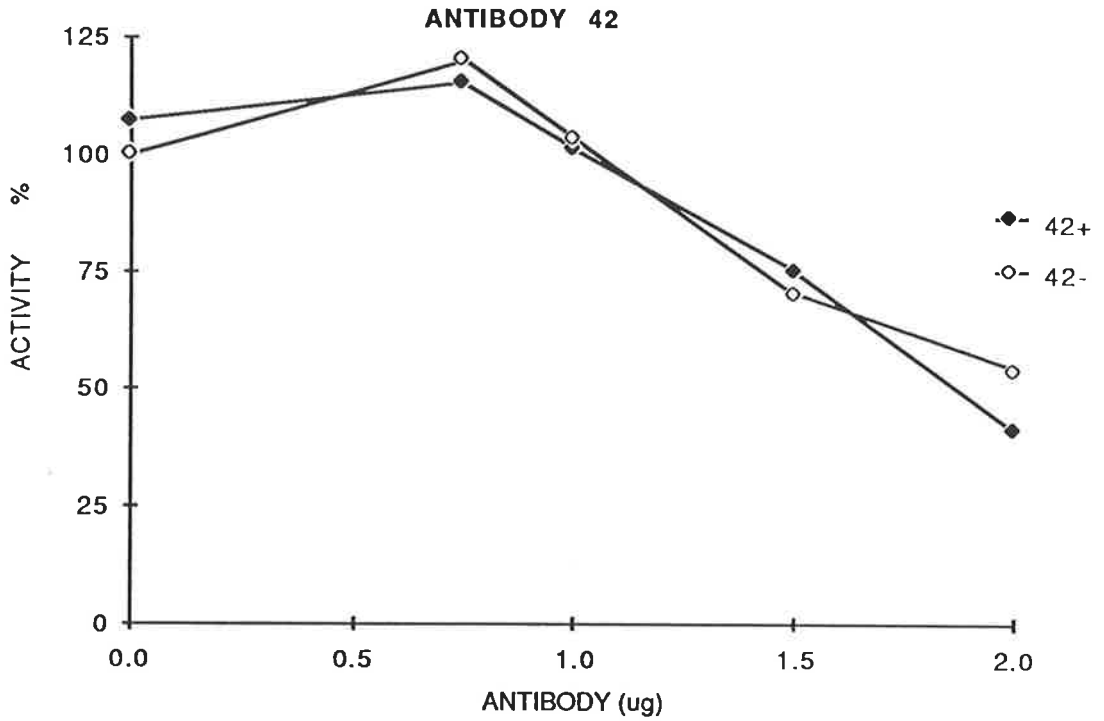

ANTIBODY 12
125
100
75
50
25
0
ACTIVITY %
0.0
0.5
1.0
1.5
2.0
ANTIBODY (ug)
12+
12-

Figure 4.6 Effect of Biotin on the Inhibition of Enzyme Activity by Monoclonal Antibodies 42 and 113

Monoclonal antibodies 42 and 113 (0-2μg) were incubated in 0.1M Tris-Cl pH 7.2 and 200μM acetyl-CoA for 30 minutes at 30°C in the presence and absence of 25 nmoles of biotin (incubation volume = 65 μl). SLPC (1.5 mUnits in 10 μl; 40 units/mg) was added to the antibodies and incubated for a further 60 minutes at 30°C. After this time the enzyme activity was determined radiochemically as described in section 3.3.2.3(b). The activity of the enzyme was expressed as a percentage of the activity of SLPC incubated without antibody or biotin. The percent activity is plotted versus the amount of antibody used.

FIGURE 4.6


ANTIBODY 42
125
100
75
50
25
0
ACTIVITY %
0.0
0.5
1.0
1.5
2.0
ANTIBODY (ug)
42+
42-

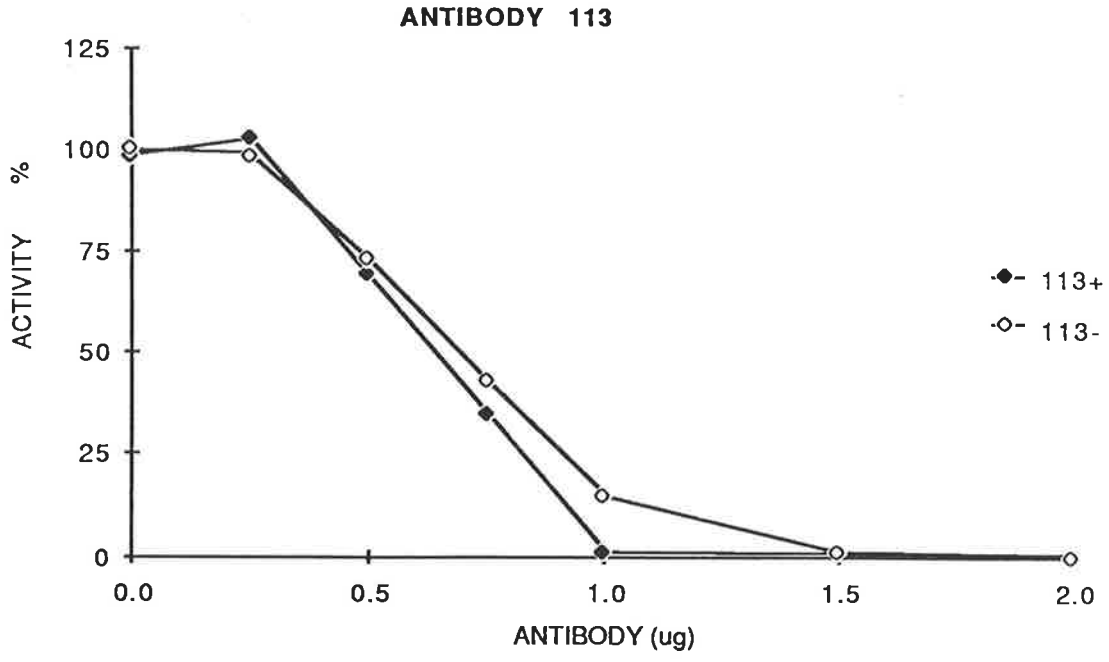

ANTIBODY 113
125
100
75
50
25
0
ACTIVITY %
0.0
0.5
1.0
1.5
2.0
ANTIBODY (ug)
113+
113-

Figure 4.7 ELISA - Monoclonal Antibodies Versus SLPC

The ELISA was carried out as described in section 2.2.10(b). The wells of a microtiter plate were coated with 50μl of SLPC (10μg/ml, specific activity = 30 units/mg) or 50μl of blocking solution (for negative controls). After blocking and washing, the wells were probed with monoclonal antibodies 6, 12 and 113 (a) and 60, 18, 42 and 80 (b) which had been purified from ascites fluid by Protein-A sepharose chromatography. Six, tenfold dilutions of each antibody were used in triplicate. The concentration of undiluted antibody was 1mg/ml. For antibody 18, the highest concentration of antibody available was 60μg/ml. The second antibody used was horseradish peroxidase conjugated rabbit anti-mouse immunoglobulin (Nordic) diluted 1,000 times. The average absorbance at 450nm of the substrate solution in triplicate wells for each antibody at each dilution was calculated. The difference between the average absorbance of the test and the control wells for each dilution of each antibody is plotted against the dilution of antibody used. For dilutions of the antibody where the absorbance at 450nm was outside the limits of the ELISA reader ($A_{450nm} \leqslant 2$), no points were plotted. SLPC = sheep liver pyruvate carboxylase.

FIGURE 4.7 a

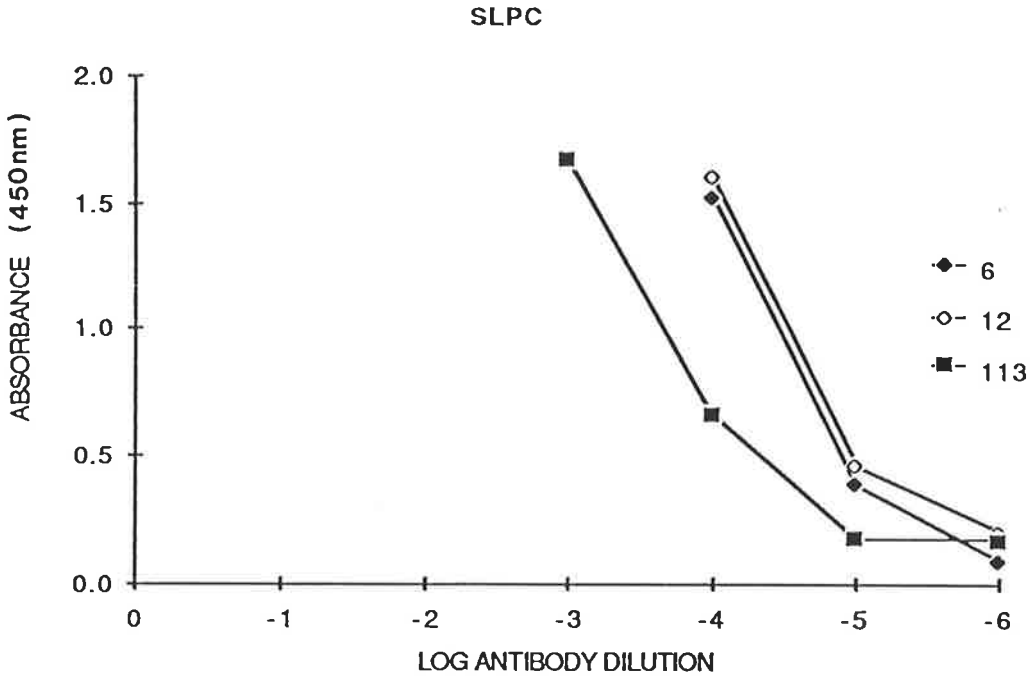


FIGURE 4.7 b

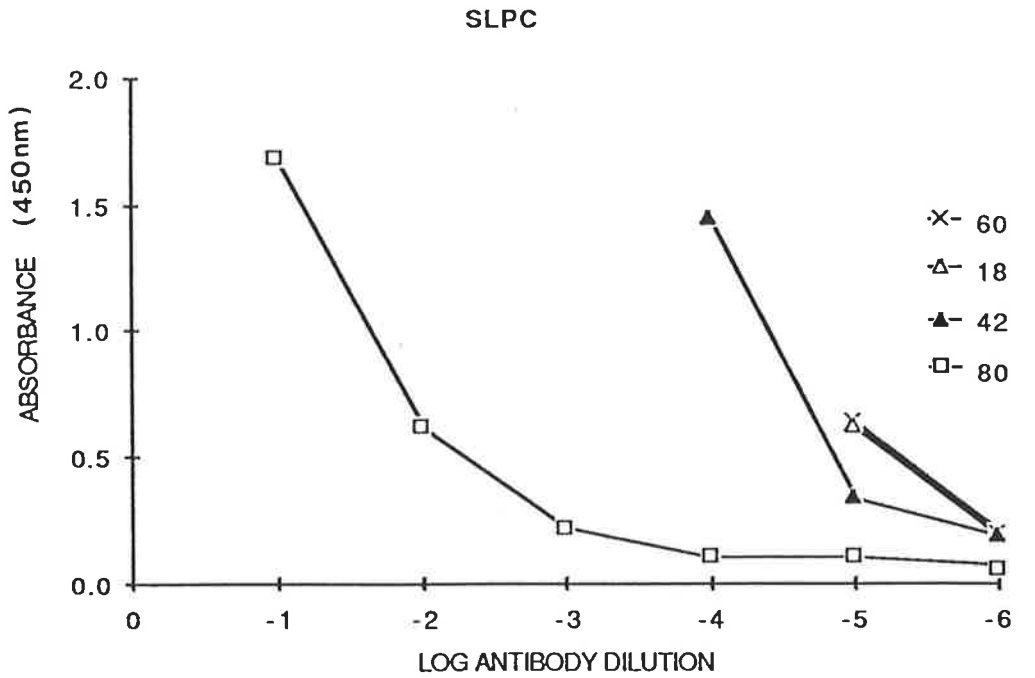


Figure 4.8 ELISA - Monoclonal Antibodies Versus CLPC

The ELISA was carried out as described in section 2.2.10(b). The wells of a microtiter plate were coated with 50μl of CLPC (10μg/ml, specific activity = 33 units/mg) or 50μl of blocking solution (for negative controls). After blocking and washing, the wells were probed with monoclonal antibodies 6, 12 and 113 (a) and 60, 18, 42 and 80 (b) which had been purified from ascites fluid by Protein-A sepharose chromatography. Six, tenfold dilutions of each antibody were used in triplicate. The concentration of undiluted antibody was 1mg/ml. For antibody 18, the highest concentration of antibody available was 60μg/ml. The second antibody used was horseradish peroxidase conjugated rabbit anti-mouse immunoglobulin (Nordic) diluted 1,000 times. The average absorbance at 450nm of the substrate solution in triplicate wells for each antibody at each dilution was calculated. The difference between the average absorbance of the test and the control wells for each dilution of each antibody is plotted against the dilution of antibody used. For dilutions of the antibody where the absorbance at 450nm was outside the limits of the ELISA reader ($A_{450nm} \leqslant 2$), no points were plotted. CLPC = chicken liver pyruvate carboxylase.

FIGURE 4.8a

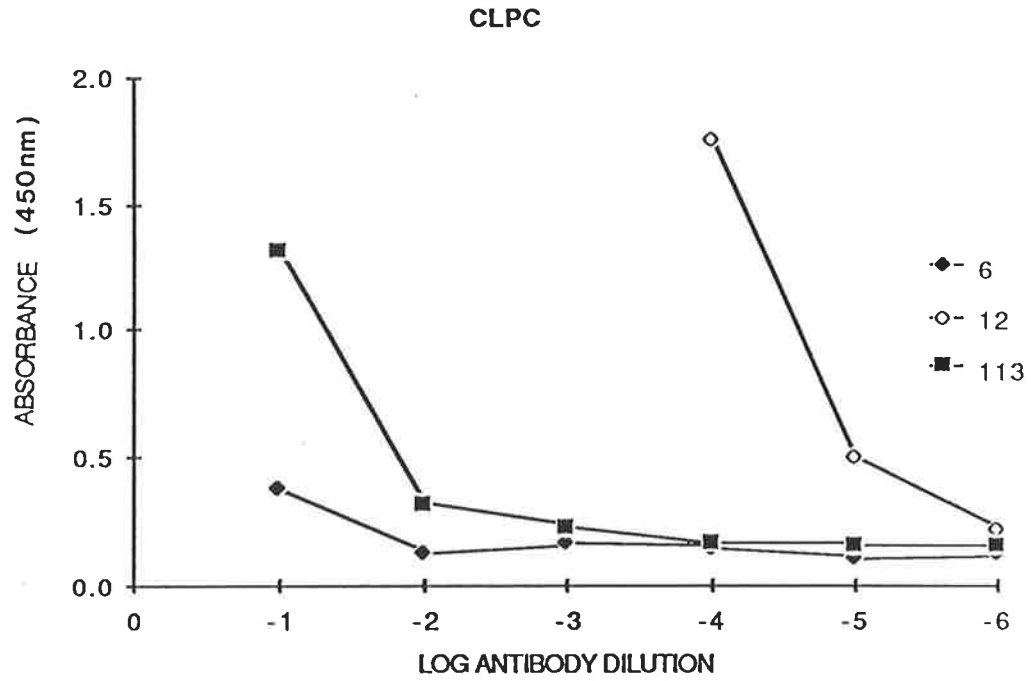


FIGURE 4.8b

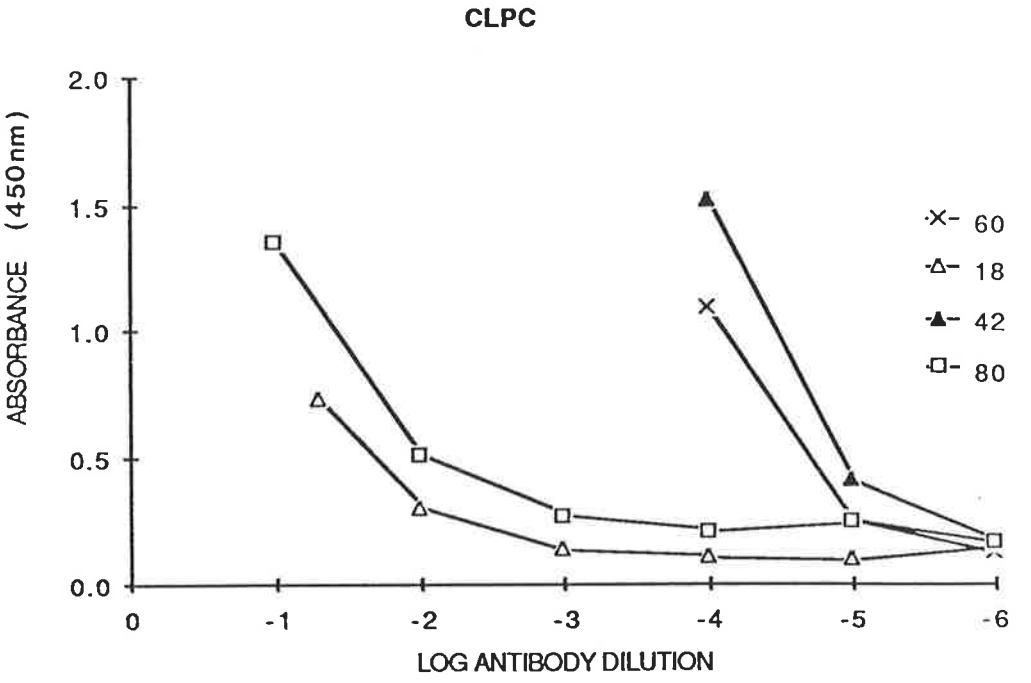


Figure 4.9 ELISA - Monoclonal Antibodies Versus Yeast Pyruvate Carboxylase

The ELISA was carried out as described in section 2.2.10(b). The wells of a microtiter plate were coated with 50μl of yeast pyruvate carboxylase (10μg/ml, specific activity = 19 units/mg) or 50μl of blocking solution (for negative controls). After blocking and washing, the wells were probed with monoclonal antibodies 6, 12 and 113 (a) and 60, 18, 42 and 80 (b) which had been purified from ascites fluid by Protein-A Sepharose chromatography. Six, tenfold dilutions of each antibody were used in triplicate. The concentration of undiluted antibody was 1mg/ml. For antibody 18, the highest concentration of antibody available was 60μg/ml. The second antibody used was horseradish peroxidase conjugated rabbit anti-mouse immunoglobulin (Nordic) diluted 1,000 times. The average absorbance at 450nm of the substrate solution in triplicate wells for each antibody at each dilution was calculated. The difference between the average absorbance of the test and the control wells for each dilution of each antibody is plotted against the dilution of antibody used. For dilutions of the antibody where the absorbance at 450nm was outside the limits of the ELISA reader ($A_{450nm} \leqslant 2$), no points were plotted. YPC = yeast pyruvate carboxylase.

FIGURE 4.9a

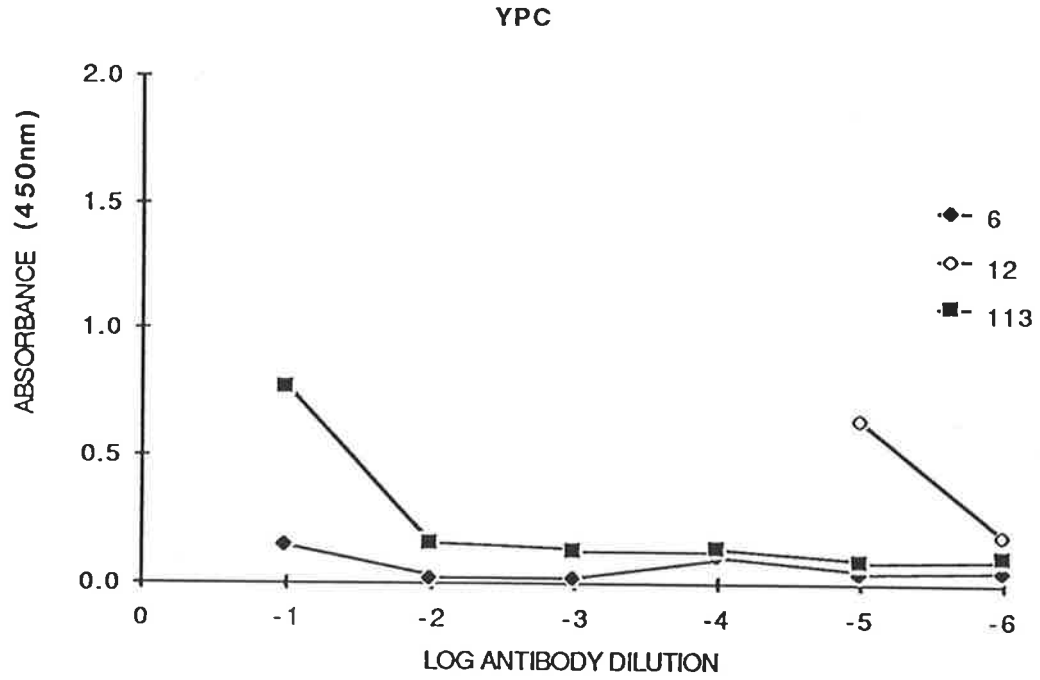


FIGURE 4.9b

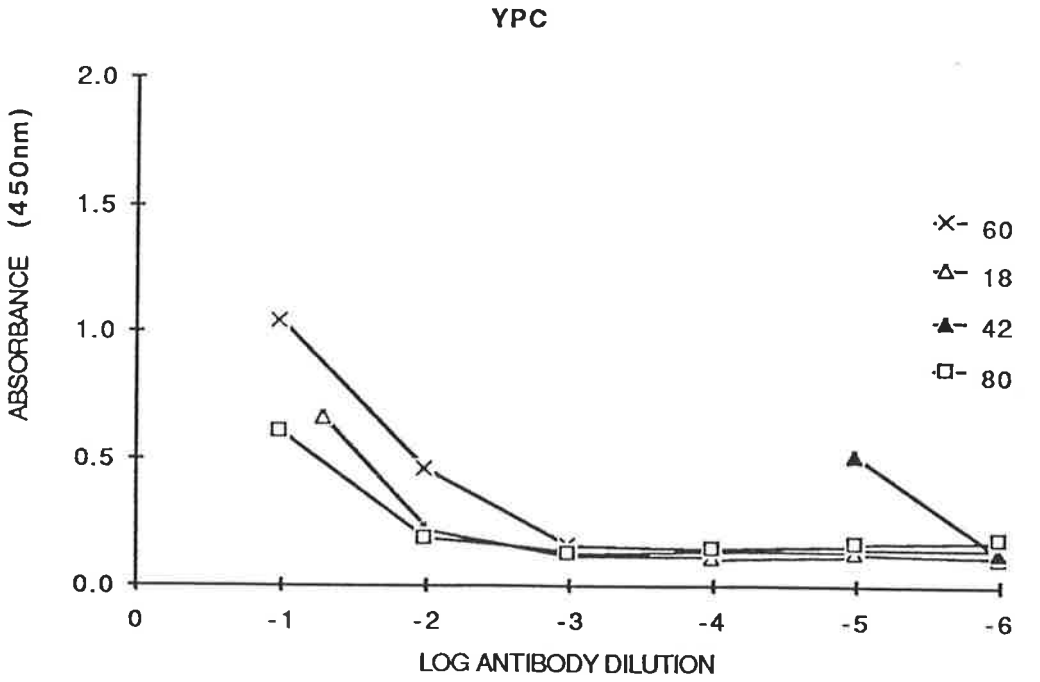


Figure 4.10 ELISA - Monoclonal Antibodies Versus Transcarboxylase

The ELISA was carried out as described in section 2.2.10(b). The wells of a microtiter plate were coated with 50μl of transcarboxylase from P. shermanii (10μg/ml) or 50μl of blocking solution (for negative controls). After blocking and washing, the wells were probed with monoclonal antibodies 6, 12 and 113 (a) and 60, 18, 42 and 80 (b) which had been purified from ascites fluid by Protein-A sepharose chromatography. Six, tenfold dilutions of each antibody were used in triplicate. The concentration of undiluted antibody was 1mg/ml. For antibody 18, the highest concentration of antibody available was 60μg/ml. The second antibody used was horseradish peroxidase conjugated rabbit anti-mouse immunoglobulin (Nordic) diluted 1,000 times. The average absorbance at 450nm of the substrate solution in triplicate wells for each antibody at each dilution was calculated. The difference between the average absorbance of the test and the control wells for each dilution of each antibody is plotted against the dilution of antibody used. For dilutions of the antibody where the absorbance at 450nm was outside the limits of the ELISA reader ($A_{450nm} \leqslant 2$), no points were plotted. TC = transcarboxylase.

FIGURE 4.10a

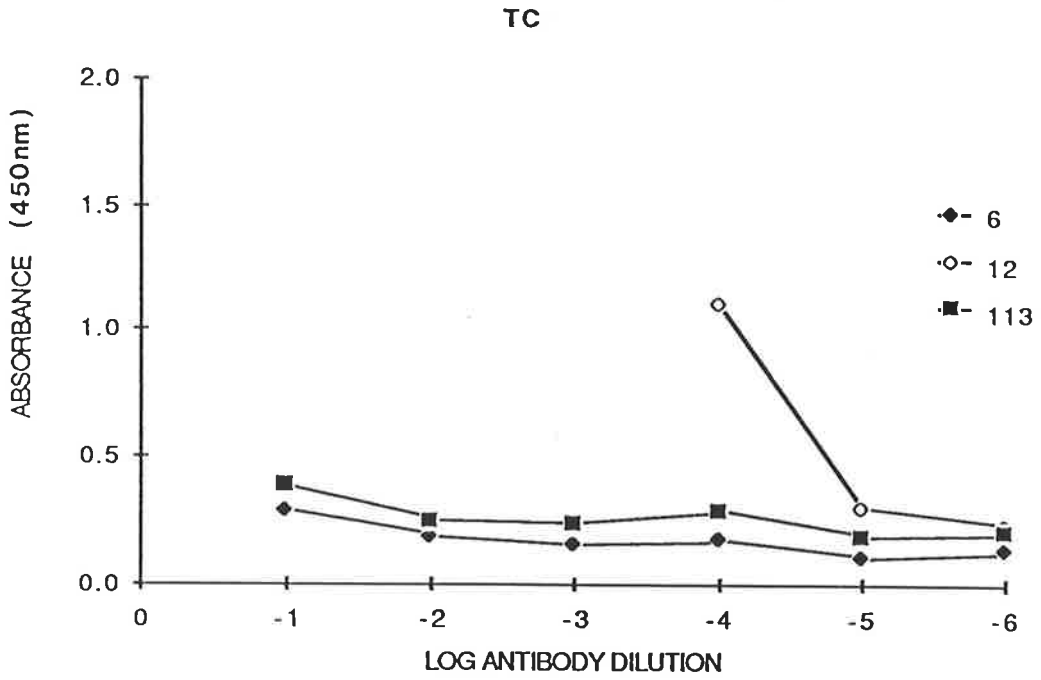


FIGURE 4.10b

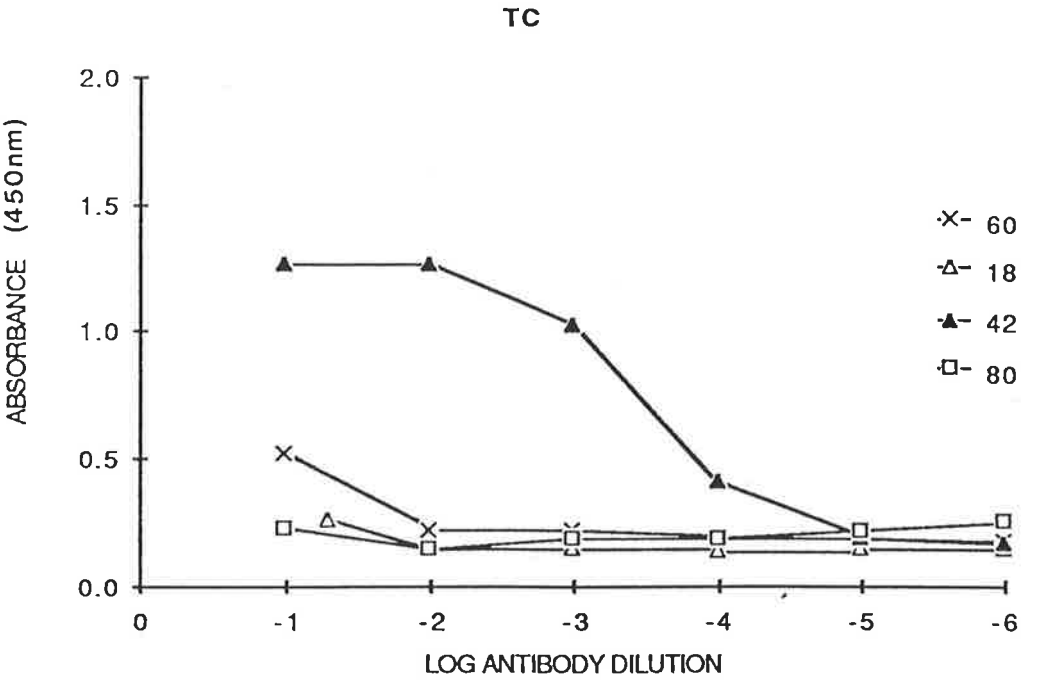


Figure 4.11 ELISA - Monoclonal Antibodies Versus SLPCC

The ELISA was carried out as described in section 2.2.10(b). The wells of a microtiter plate were coated with 50μl of sheep liver propionyl-CoA carboxylase (10μg/ml) or 50μl of blocking solution (for negative controls). After blocking and washing, the wells were probed with monoclonal antibodies 6, 12 and 113 (a) and 60, 18, 42 and 80 (b) which had been purified from ascites fluid by Protein-A sepharose chromatography. Six, tenfold dilutions of each antibody were used in triplicate. The concentration of undiluted antibody was 1mg/ml. For antibody 18, the highest concentration of antibody available was 60μg/ml. The second antibody used was horseradish peroxidase conjugated rabbit anti-mouse immunoglobulin (Nordic) diluted 1,000 times. The average absorbance at 450nm of the substrate solution in triplicate wells for each antibody at each dilution was calculated. The difference between the average absorbance of the test and the control wells for each dilution of each antibody is plotted against the dilution of antibody used. For dilutions of the antibody where the absorbance at 450nm was outside the limits of the ELISA reader ($A_{450nm} \leqslant 2$), no points were plotted. SLPCC = sheep liver propionyl-CoA carboxylase.

FIGURE 4.11 a

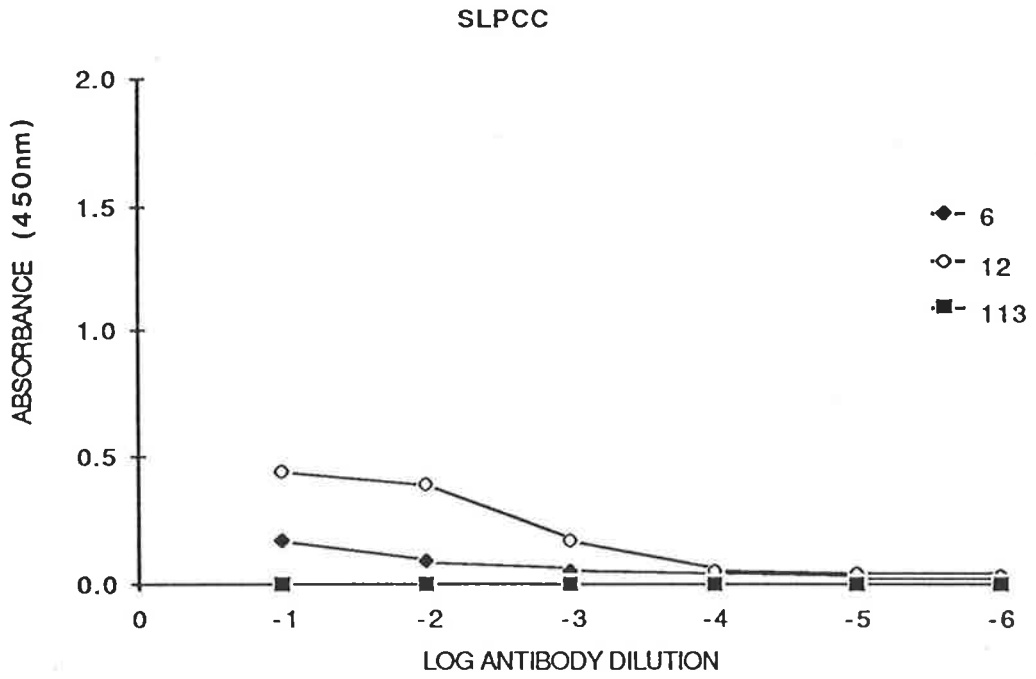


FIGURE 4.11 b

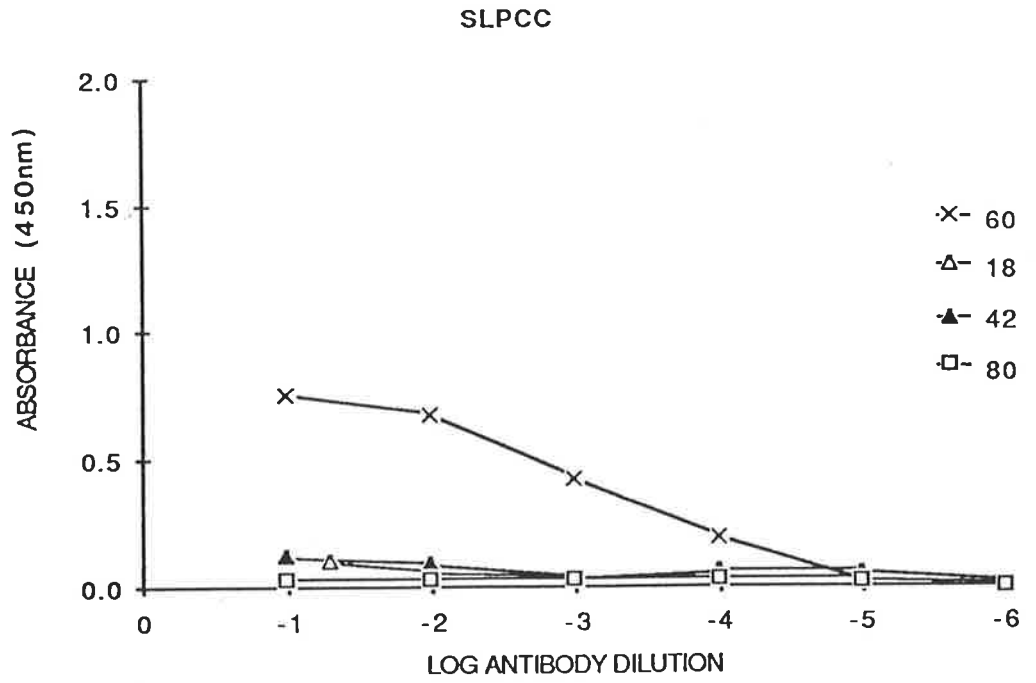


Figure 4.12 Models and Schematic Representation of Pyruvate Carboxylase Complexed with Avidin

(a) Model of avidin complexed to pyruvate carboxylase in a chain-like structure as would be observed at ratios of avidin : enzyme between 2:1 and 1:2. Each enzyme tetramer is connected to the next via avidin molecules (hatched) positioned on the external faces of the enzyme tetramer (i.e. on opposite sides of the enzyme tetramer).

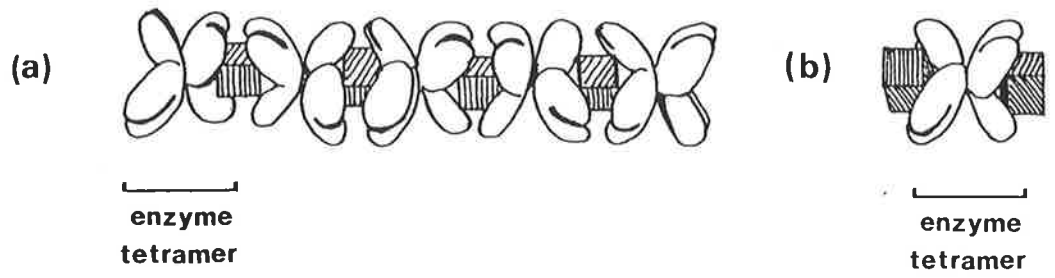
(b) Model of a single tetramer complexed with avidin (hatched) as would be observed at ratios of avidin : enzyme of 10:1. Like the chain structure, the avidin molecules bind to the external faces of the enzyme tetramer close to the intersubunit junction.

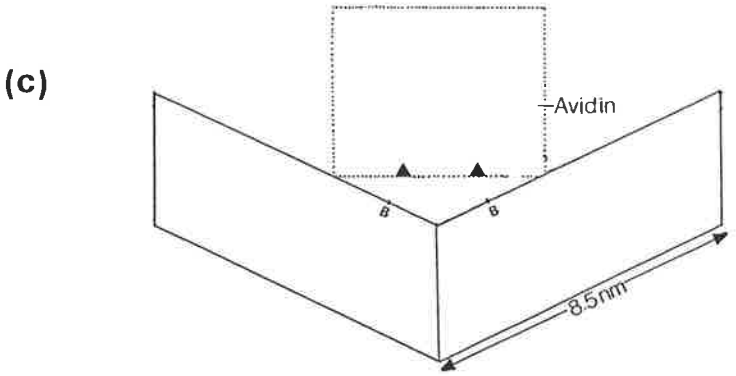
(c) Diagrammatic representation of a side view of half an enzyme tetramer with avidin bound to an external face.
The biotin binding sites on avidin (▲) and the biotin prosthetic group on the enzyme (B) are indicated.

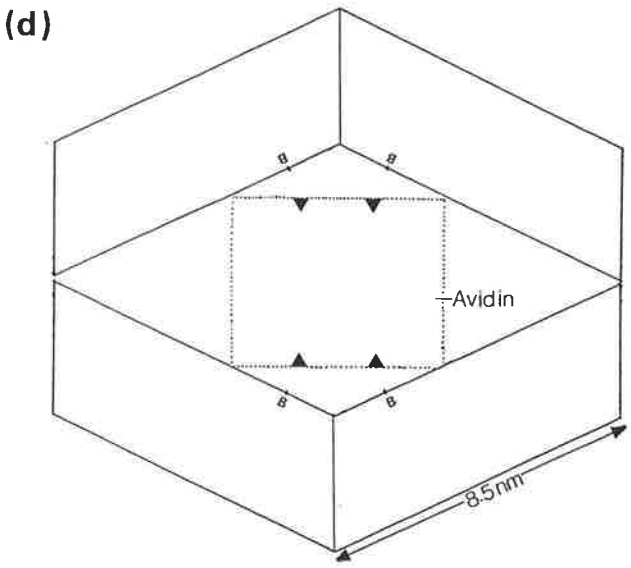
(d) Diagrammatic representation of dimers from two adjacent tetramers of pyruvate carboxylase in an enzyme : avidin chain, encompassing avidin by their external faces.

(e) An IgG molecule showing the Fc and two Fab regions has been drawn to scale.

FIGURE 4.12


(a)
enzyme
tetramer
(b)
enzyme
tetramer


(c)
Avidin
B
B
8.5nm


(d)
B
B
Avidin
B
B
8.5nm

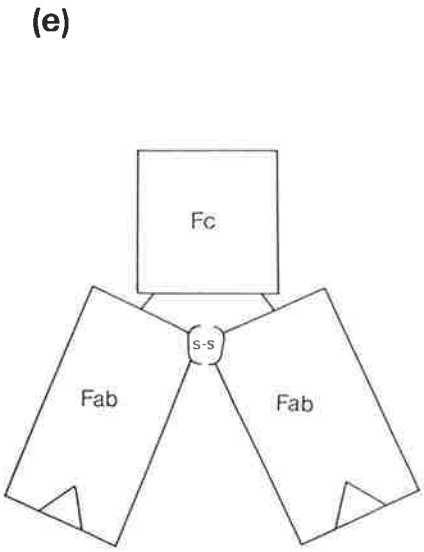

(e)
Fc
S-S
Fab
Fab

Figure 4.13 Schematic Representation of Pyruvate Carboxylase Interacting with Anti-Biotin IgG, Fab and Avidin

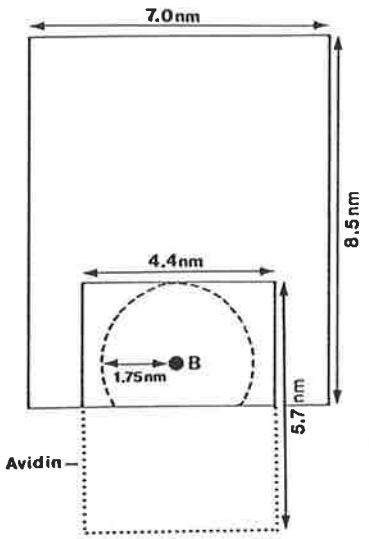
(a) Diagrammatic representation of a subunit of pyruvate carboxylase of dimensions 8.5 x 7.0 nm (top view) showing the position of the biotin prosthetic group (B) and the area occluded by avidin (5.7 x 4.4 nm) and by the end of an anti-biotin antibody arm, 3.5 nm in diameter (Tzartos et al., 1981) bound to the enzyme subunit.

(b) Side view of a dimer of pyruvate carboxylase with an avidin tetramer attached or an anti-biotin Fab fragment bound to a biotin prosthetic group (B) on one subunit of the dimer. The contact region of the antigen combining end of one Fab domain is similar to that of half an avidin tetramer which would bind in this region on one enzyme subunit.

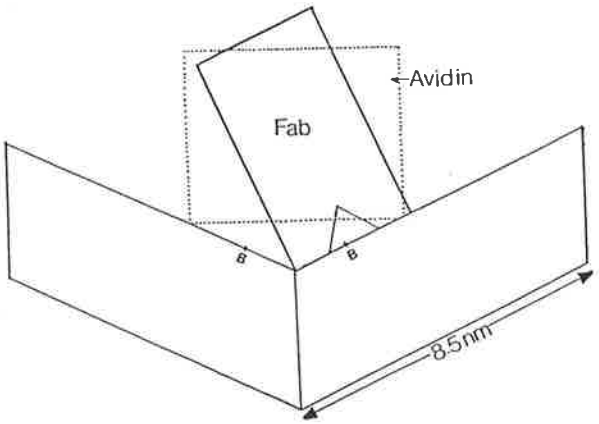
(c) The full anti-biotin IgG molecule attached to one subunit of a dimer of pyruvate carboxylase. Steric hindrance caused by the towering superstructure of this molecule must effectively enlarge the area excluded by the IgG molecule over that of its Fab fragment.

FIGURE 4.13

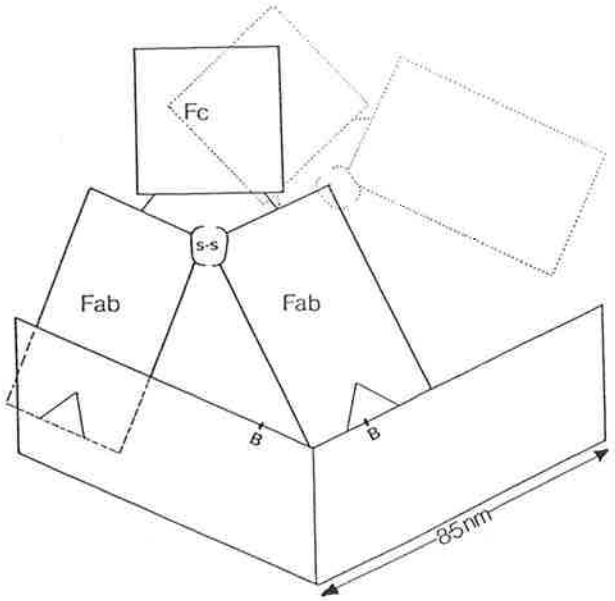
(a)



(b)



(c)



CHAPTER 5

ELECTRON MICROSCOPY

5.1 INTRODUCTION

Having performed the initial biochemical characterisation of the monoclonal antibodies against sheep liver pyruvate carboxylase, the question arose as to the position of binding of these antibodies on the enzyme tetramer. Using electron microscopy, Mayer *et al*. (1980) established the quarternary structure of pyruvate carboxylase from chicken, sheep and rat liver. In addition Johannssen *et al*. (1983) used electron microscopy and avidin to ascertain the position of the biotin prosthetic group and thus the active site of chicken liver pyruvate carboxylase as discussed in Chapter 4. Hence, this technique has proved, so far, to be an extremely powerful tool in determining the fine structure of pyruvate carboxylase in the absence of crystallographic data.

Therefore, it was decided to determine the position of binding of the monoclonal antibodies and a polyclonal antibody to sheep liver pyruvate carboxylase by electron microscopy. The antibodies used in this study were monoclonal antibodies 6, 12, 18, 42, 60, 80 and 113 and an anti-biotin antibody of polyclonal origin. All of the antibodies tested were of the IgG subclass. SLPC was incubated with intact monoclonal antibodies or Fab fragments of the anti-biotin antibodies and the immune complexes were isolated using HPLC gel filtration. To determine the region of attachment of the antibody molecule or Fab fragment to SLPC, the complexes were analysed by electron microscopy.

Johannssen *et al*. (1983) were able to visualise avidin bound to chicken liver pyruvate carboxylase under the electron microscope. Knowing dimensions of both pyruvate carboxylase and the avidin tetramer as well as the position of the four biotin binding sites on the avidin tetramer, they were able to determine the area of the enzyme bound by avidin and to localise the biotin prosthetic group on the enzyme to within 3 nm of the intersubunit junction. A diagrammatic representation of the position of

avidin binding and the biotin prosthetic group on one subunit of a pyruvate carboxylase tetramer is shown in Figure 5.9. Also indicated is the area on the enzyme subunit that is covered by an immunoglobulin bound at the biotin site. This was calculated from the dimensions of an IgG molecule determined from X-ray diffraction data (Sarma et al. 1971).

The electron microscopic studies described in this chapter combined with the competitive binding and enzyme inhibition data from Chapter 4 were utilised to physically locate the binding position of anti-biotin antibodies and some of the monoclonal antibodies to sheep liver pyruvate carboxylase. The work with the anti-biotin Fab fragments was undertaken in order to confirm the position of the biotin prosthetic group on SLPC as determined by Johannssen et al. (1983) using avidin on CLPC, and to confirm that Fab fragments attached to SLPC could be resolved by electron microscopy.

The electron microscopy undertaken in this chapter was carried out in collaboration with visiting scientists from the University of Göttingen, Professor F. Mayer and Dr Manfred Rohde.

5.2 METHODS AND MATERIALS

5.2.1 Preparation of Rabbit Anti-Biotin IgG Fab Fragments

Antiserum to biotin was prepared by immunisation of a rabbit with biotinylated thyroglobulin. The immune serum (5ml) was diluted with 0.1M sodium phosphate pH 8.0 (2ml) and loaded onto a Protein A-Sepharose column (volume 5ml) equilibrated in 0.1M sodium phosphate pH 8.0 at room temperature. The column was washed with 30ml of the phosphate buffer until the absorbance at 280 nm was zero. The immunoglobulin fraction was then eluted with 0.1M sodium citrate pH 3.0 and neutralised immediately with 1M Tris.

The immunoglobulin fraction was concentrated then dialysed extensively against 0.1M sodium acetate pH 5.5. Immunoglobulin at 3mg/ml in 0.1M sodium acetate, 1 mM DTE and 2 mM EDTA pH 5.5 was digested with papain for 6 hours at 37°C such that the concentration of papain was 1% w/v. The reaction was stopped by dialysis against water.

Fab fragments were separated from Fc fragments and undigested IgG molecules by filtration on CM-Sepharose CL-6B as described by Porter (1959). Fab fraction II was used in the subsequent experiments.

5.2.2 Preparation of SLPC-anti-biotin Fab Complexes

Sheep liver pyruvate carboxylase (50μg, s.a. = 30 units/mg) in 0.1M Tris-Cl, 0.1M KCl, pH 7.2 was incubated with 250μg of anti-biotin Fab fragments in the same buffer at 30°C for one hour. This gave a 25 times molar excess of Fab fragments over SLPC subunits (Note however, that the Fab fragments originated from a polyclonal serum and that only a small fraction of these would have been directed towards the biotin moiety). The mixture was loaded onto an HPLC gel filtration column (TSK G4000 SW) which had been equilibrated with 0.1M Tris-Cl, 0.1M KCl, 100μM acetyl-CoA, pH 7.2 at a flow rate of 0.1ml min^{-1}. Protein eluting from the column was detected as a wavelength of 215 nm.

5.2.3. Crosslinking of SLPC with dithio-bis-(succinimidyl propionate)

Sheep liver pyruvate carboxylase (27 units/mg) which had been purified by avidin-sepharose chromatography was crosslinked with dithio-bis-(succinimidyl propionate) (DTSP) following a modified method of Loman and Fairbanks (1976). DTSP (10 mM in 63% acetone) was added slowly to SLPC in 0.1M sodium phosphate pH 7.0, such that the final

concentration of the enzyme and DTSP was 1mg/ml and 1 mM respectively. The reaction was stopped after ten minutes at 25°C by the addition of lysine to a final concentration of 40 mM.

5.2.4 Monoclonal Antibodies

Monoclonal antibodies used in the binding studies had been isolated from ascites fluid by a 50% ammonium sulphate precipitation and dialysed against 0.1M sodium phosphate pH 7.0. Monoclonal antibodies 6, 12, 18, 42, 60, 80 and 113 were used. The titres of antibodies 78 and 98 in ascites fluid were considered to be too low and were not used.

5.2.5 Preparation of SLPC-Monoclonal Antibody Complexes

Sheep liver pyruvate carboxylase (100μg crosslinked with DTSP) in 0.1M sodium phosphate pH 7.0 was incubated with 500μg of monoclonal antibodies 6, 18, 60, 80 and 113 (4x molar excess of antibody over enzyme subunits), 250μg of antibody 12 (2 x molar excess of antibody over enzyme subunits) and 200μg of antibody 42 (1.6x molar excess) in the same buffer for half an hour at 37°C. After this time, all of the mixture was chromatographed on an HPLC gel filtration column (TSK G3000 SW) equilibrated with 0.1M Tris-Cl, 0.1M KCl pH 7.2 at a flow rate of 0.5ml min^{-1}. Fractions were collected every minute in order to separate unbound antibody and enzyme from the antibody/enzyme complex. Protein eluted from the column in the same buffer was detected at a wavelength of 280 nm.

5.2.6 Electron Microscopy

The electron microscopy described in this chapter was carried out by Professor Frank Mayer and Dr. Manfred Rohde.

Thin carbon support films, approximately 4-6 nm thick, were prepared by indirect sublimation of carbon onto freshly cleaned mica. Samples of sheep liver pyruvate carboxylase - monoclonal antibody complexes, SLPC-anti-biotin Fab complexes, anti-biotin Fab and SLPC alone were provided at final enzyme concentrations in the range of 50-100µg/ml. Using 400 mesh copper grids, these samples were negatively stained with 4% w/v aqueous uranyl acetate pH 4.5 according to the method of Valentine et al. (1968). Specimens were examined and micrographs taken with a Philips EM300 electron microscope at an acceleration voltage of 80kV using Kodak Electron Microscope Film 4489. Magnifications were calibrated using a lined grating replica. The primary magnification was 51200x.

5.3 RESULTS AND DISCUSSION

5.3.1 Preparation of SLPC-anti-biotin Fab Complexes

Figure 5.1 shows the gel filtration elution profiles of SLPC (a), anti-biotin Fab fragments (b) and SLPC mixed with Fab fragments (c) as described in section 5.2.2. For these experiments, 100 µM acetyl-CoA was included in the column running buffers to prevent inactivation by dilution and to preserve the tetrameric structure of the enzyme. (100 µM acetyl-CoA had previously been shown to be sufficient for this purpose - Y.S. Khew, personal communication.) Although some satisfactory micrographs were obtained in these experiments, well preserved tetramers of SLPC were not observed consistently. Despite the flow rate being was

extremely slow (0.1ml min^{-1}), the resolution of the column was such that two separate high molecular weight protein peaks containing SLPC alone in one and the enzyme with Fab fragments attached in the other, were never observed when the SLPC-Fab mixture was subject to gel-filtration. Protein peaks emerged at about 77 minutes and 100 minutes which corresponds to SLPC alone and Fab fragments alone respectively. Fractions which were collected around the 77 minute peak were mounted for electron microscopy. SLPC alone in 250 μM acetyl-CoA and anti-biotin Fab fragments were also mounted as described in section 5.2.6.

5.3.2 Analysis and Discussion of Electron Micrographs of SLPC-Anti-Biotin Fab Complexes

Figures 5.2 (a) and (b) show samples of negatively stained SLPC incubated prior to mounting in the presence of 250 μM acetyl-CoA. Three different projections of the SLPC tetramer are shown. Figure 5.2 (c) shows pure IgG Fab fragments. Two protein masses in each fragment are clearly visible. In 5.2 (d) SLPC-Fab complexes in two different orientations are pictured. The upper row shows complexes where there is a triangular projection of the enzyme while in the lower row side on views can be observed. It is evident that as well as the enzyme protein masses (filled arrowheads) additional protein masses are present (open arrow heads) which correspond to the size of the isolated Fab fragments and are not visible when SLPC alone is mounted for electron microscopy. These complexes are represented diagramatically in Figure 5.2 (e) where the SLPC subunits are the solid black areas while the Fab fragments are shown by contours only. A complex containing two Fab fragments (arrowed) is shown in the upper row.

The Fab fragments were always seen to attach close to the centres of the enzyme tetramer rather than at the ends of the subunit. This observation is in agreement with the findings obtained for the location of biotin groups on pyruvate carboxylase from chicken liver (Johannssen et al., 1983), Aspergillus nidulans (Osmani et al., 1984) Rhizopus arrhizus (Mayer et al., 1985) and Saccharomyces cerevisiae (Rohde, et al., 1985) by electron microscopic examination of pyruvate carboxylase-avidin complexes. The biotin prosthetic groups on the pyruvate carboxylase studied appear to be located on the external faces of the enzyme subunits close to the intersubunit junction. It is interesting that more than two Fab fragments per SLPC tetramer were never observed. Possibly the binding of the anti-biotin Fab to one of the two subunits in a dimer sterically prevents the binding of another Fab to the other subunit in that dimer. This means that another Fab could only bind to an enzyme subunit on the opposite face of the enzyme and thus would not be readily observed.

5.3.3 Crosslinking of SLPC with DTSP

To assist in the analysis of electron micrographs of enzyme-antibody complexes it was necessary to separate unbound antibody and enzyme from the enzyme antibody complexes. High performance gel filtration was chosen for the separations. A disadvantage in using this method was that the dilution of the enzyme during gel filtration resulted in loss of structure, especially dissociation of tetramers. It is known that SLPC undergoes irreversible inactivation when diluted to concentrations below 4units/ml (Ashman et al., 1972) and that dissociation of the enzyme tetramers into dimers and monomers also occurs.

Acetyl-CoA is known to prevent inactivation by dilution and preserve the tetrameric structure of the intact enzyme (Ashman et al.,

1972, Mayer et al., 1980). Gel filtration was performed where acetyl-CoA 100 uM was included in the running buffers (see sections 5.3.1 and 5.3.2). This did not prove to be successful however in consistently obtaining intact enzyme tetramer:antibody complexes.

To overcome this problem, intramolecular crosslinking of the SLPC subunits with DTSP before incubation with antibody and subsequent gel filtration, was performed (see section 5.2.3). Intramolecular crosslinking would prevent the dissociation of SLPC into dimers and monomers caused by dilution during the gel filtration step. Previously this procedure was successfully employed in the separation of SLPC-anti-TNP-Fab complexes (A. Chapman-Smith, 1981).

To test the success of the crosslinking, samples were diluted in the absence of acetyl-CoA and compared by electron microscopy to the same enzyme which was not crosslinked and diluted where no acetyl-CoA was present (see Figure 5.3). It was found that modification of SLPC for only ten minutes with DTSP yielded a high percentage of intact tetramers after dilution in the absence of acetyl-CoA. Conversely, the same enzyme that was not crosslinked and diluted in the absence of acetyl-CoA showed many broken enzyme particles and few intact tetramers. This indicated that the intramolecular crosslinking was successful and the crosslinked preparation of SLPC was considered to be suitable for complexing with monoclonal antibodies.

5.3.4 Preparation of SLPC-Monoclonal Antibody Complexes

A TSK G3000 SW HPLC column was calibrated with the following molecular weight standards: thyroglobulin (M_r=669,000), ferritin (M_r=440,000), glutamate dehydrogenase (M_r=330,000), pyruvate kinase (M_r=237,000), aldolase (M_r=158,000), IgG (M_r=150,000), glutamate oxaloacetate transaminase (M_r=95,000), malic dehydrogenase

(M_r=70,000) and ovalbumin (M_r=45,000). The calibration curve is shown in Figure 5.4. The curve appears to be non-linear for the high molecular weight standards (ie. greater than 400,000).

When SLPC which has been intramolecularly crosslinked with DTSP is chromatographed in this system protein peaks emerge after approximately 17.5, 21 and 28 minutes (see Figure 5.5[a]). Material from the 17.5 minute fraction when observed under the electron microscope appears to consist of tetrameric particles of SLPC. This elution time is also consistent with the elution time of catalytically active tetrameric SLPC on this column observed by other members of the laboratory. The material in the other peaks was not analysed by electron microscopy. However, from the calibration curve for this column the peak at 28 minutes would have a molecular mass of 120 kDa which is the approximate molecular mass of the SLPC monomer. Dimers of SLPC (~240 kDa molecular mass) would be expected to elute at 25 minutes. The composition of protein in the peak eluting at 21 minutes was not investigated. It appeared then that tetramers of pyruvate carboxylase with a molecular mass of approximately 480 kDa did not run in the linear part of the calibration curve. Hence the elution time of SLPC tetramer:antibody complexes could not be predicted.

Figures 5.5(a) and (b) show the elution profiles of SLPC which had been incubated with monoclonal antibodies 6, 12, 18, 42, 60, 80 and 113 as described in section 5.2.5. For all of the antibodies the first peak eluted at 17-17.75 minutes. Electron microscopic examination of fractions from this first eluting peak showed SLPC-antibody complexes. Many enzyme molecules without antibody attached were also present. In addition some dimers and monomers of SLPC were evident indicating that some enzyme tetramers had not been stabilised by crosslinking and thus dissociation occurred during mounting for microscopy. Two separate protein peaks containing high molecular weight material with enzyme

tetramers alone in one peak and enzyme tetramers with IgG attached in another were never observed. Protein peaks were often observed eluting at approximately 26 and 28-29 minutes but electron microscopic examination of fractions from these peaks for each antibody was not carried out. It is possible that these peaks contained respectively, free unbound IgG antibody of molecular mass 150 kDa and enzyme monomers of molecular mass 110-120 kDa. Representative micrographs from the first eluting protein peak for each antibody are shown in Figures 5.6-5.8.

5.3.5. Analysis of Electron Micrographs of SLPC-Monoclonal Antibody Complexes

Antibody 6 (Figure 5.6) appeared to bind close to the central part of the enzyme tetramer, that is, close to the intersubunit junction. For most enzyme-antibody complexes only one antibody bound per enzyme tetramer suggesting that the binding of one antibody to an enzyme tetramer would sterically hinder the binding of another in most cases. Depending on the orientation of the epitopes with respect to each other, this may be expected of an antibody which is binding close to the central region of the molecule.

In Figure 5.6 micrographs of some SLPC-antibody 12 complexes are also displayed. Like antibody 6, the molecule also binds at the central part of the enzyme tetramer, however this antibody almost always lies completely over the intersubunit junction. In most cases [except (i)] only one antibody binds per enzyme tetramer which suggests that steric hindrance prevents more antibodies from binding in the central region of the molecule.

Micrographs of some SLPC-antibody 18 complexes are also shown in Figure 5.6. Antibody 18 appears to bind to the outer parts of the enzyme subunits, ie. distal to the intersubunit junction.The antibody molecule

was frequently seen protruding from the ends of the subunits of the enzyme. Often more than one antibody molecule was seen to bind to the enzyme tetramer. Since all available evidence indicates that a tetramer of SLPC is comprised of four identical subunits, then it follows that up to four antibody molecules could bind per tetramer providing this is sterically possible. If the site for binding is at the ends of the subunits then steric hindrance caused by the binding of one antibody is unlikely to occur.

Figure 5.7 shows a selection of micrographs of SLPC-antibody 42 complexes. Like antibody 12, this molecule appears to bind extremely close to, or over the intersubunit junction at the centre of the molecule. In most cases only one antibody molecule bound to the enzyme tetramer which suggests that the antibody binding sites on the enzyme are so close that the binding of one antibody sterically hinders the binding of another. Molecules of antibody 42 were rarely seen "protruding" from the tetramer. They were mostly seen to lie over the central region of the tetramer.

Micrographs of SLPC-antibody 60 complexes are also displayed in Figure 5.7. Like antibody 18, this antibody appears to bind close to the ends of the enzyme subunits in the tetramer. However, few of the antibody molecules "protrude" out from the molecule but rather seem to lie over the enzyme tetramer. Hence it is possible that the binding site for antibody 60 may not be as distal from the intersubunit junction as the binding site for antibody 18. Frequently more than one antibody molecule was seen to bind to the enzyme tetramer again suggesting a binding site distal to the central part of the molecule.

Greater than one antibody 80 molecule bound per SLPC tetramer was often observed which suggests that the antibody does not bind close to the intersubunit junction (See Figure 5.7). The antibodies did not appear to bind at the ends of the subunits and only seldomly at the

central part of the enzyme molecule. Hence a position of antibody 80 binding was assigned midway between the intersubunit junction and the ends of the subunits on the external face.

Like antibody 6, 113 binds close to the central part of the enzyme tetramer. However, this antibody does not lie completely over the intersubunit junction as do antibodies 12 and 42. Sometimes greater than one antibody molecule was observed to bind. Antibodies 113 and 6 have very similar binding patterns to SLPC as viewed under the electron microscope. Micrographs of SLPC-antibody 113 complexes are shown in Figure 5.8.

5.3.6 Discussion of Electron Microscopy Results for SLPC-Monoclonal Antibody Complexes

Taking into account the biochemical data from previous chapters involving these antibodies as well as the electron micrographic observations shown in Figures 5.6 - 5.8, the approximate position of binding of antibodies 6, 12, 18, 42, 60, 80 and 113 on one subunit of SLPC were assigned as shown in Figure 5.9.

All of the antibodies used for electron microscopic examination (except antibody 113) were tested for their ability to bind to SLPC in the presence of avidin and goat anti-biotin antibodies (see section 4.2.2.3). Clearly there is a dramatic decrease in binding of antibodies 12 and 42 in the presence of avidin. This was also observed for these antibodies in the presence of anti-biotin antibodies. In addition, the binding of antibody 80 to SLPC was affected by anti-biotin antibodies but not by avidin. In Figure 5.9 the area on an SLPC subunit covered by both avidin and an IgG molecule binding at the biotin site are shown. The position of biotin as determined by Johannssen et al. (1983) and therefore the active site is also indicated. Clearly antibodies 12 and

42 must bind within the avidin binding site as indicated. The anti-biotin IgG molecule, being effectively much larger than the avidin molecule, excludes a larger area on the subunit available for binding to particular monoclonal antibodies (see Figure 4.13). Hence antibody 80 has been assigned to a region within the IgG exclusion area but outside the avidin binding area.

In section 4.2.2.2 the binding of antibodies 12 and 42 to CLPC was dramatically impaired when the enzyme had been previously complexed with avidin. This did not occur for antibodies 60 and 78. These results support the proposal that antibodies 12 and 42 bind within or close to the avidin binding site. (Antibodies 6, 18 and 113 were not tested in competition with avidin for CLPC.)

Antibodies 12 and 42 were also shown to inhibit both CLPC and SLPC activity. Their inhibition of SLPC activity decreased in the presence of the product oxaloacetate. In addition, the substrate $MgATP^{2-}$ caused a decrease in inhibition by antibody 42. Although they were shown by ELISA to bind to SLPC and CLPC, antibodies 6, 18 and 113 were shown to inhibit SLPC but not CLPC. The presence of substrates did not have such dramatic effects on inhibition of enzyme activity with the exception of antibody 6 where greater inhibition by the antibody was observed in the presence of oxaloacetate. It was suggested from these results that antibodies 12 and 42 bound close to the active site of both SLPC and CLPC. The fact that antibodies 6, 18 and 113 did not inhibit CLPC activity suggested that binding of these antibodies was not at the active site of the chicken enzyme. None of the inhibitors of SLPC activity were anti-biotin antibodies (see section 4.2.1.3).

Based on all of this evidence, the position of binding of antibodies 12 and 42 has been assigned within the avidin binding site close to the biotin prosthetic group and hence the active site of the molecule. The electron microscopic data supports this assignment.

Electron micrographs revealed that the binding of antibody 6 was close to the intersubunit junction but these antibodies were not seen consistently to lie completely over the centre of the tetramer as did antibodies 12 and 42. Since the binding of antibody 6 to SLPC was not excluded by avidin or anti-biotin antibodies but the antibody did inhibit SLPC (but not CLPC), the position of binding was assigned to the side of the enzyme subunit that is still close to the intersubunit junction.

The electron micrographs of antibody 113-SLPC complexes revealed the same area of binding as for antibody 6. The only biochemical data of use here in assigning a binding site for this antibody, is its inhibition of only SLPC with no effects of substrates or products on its inhibition. Experiments where exclusion of binding by avidin or anti-biotin antibodies was investigated were not performed with this antibody. Its position of binding is probably not directly over the active site of the molecule and been assigned to the side of the molecule as for antibody 6.

Antibody 80 does not inhibit pyruvate carboxylase activity. Goat anti-biotin antibodies but not avidin were shown to compete with antibody 80 for binding. Hence this antibody must bind within the anti-biotin binding site as indicated in Figure 5.9 but outside the area covered by avidin binding. Electron micrographs also indicate that this antibody binds in the middle third of the subunit (as opposed to the inner and outer thirds). In section 4.2.2.3 it was shown that the binding of antibody 80 to SLPC which had been modified by TNBS in the presence or absence of acetyl-CoA was decreased to the same extent in the presence of anti-TNP antibodies. This suggested that Lys-B is close to the area of monoclonal antibody 80 binding. Since anti-biotin antibodies competed with antibody 80 for binding, it would be expected then that Lys-B is found in the anti-biotin exclusion area as well. This is supported by the observation that competition of anti-biotin antibodies with anti-TNP

antibodies affected binding to Lys-B only. Recent electron micrographic studies have shown that anti-TNP-Fab fragments bind to the TNBS modified enzyme in the same area as antibody 80 binding (M. Rohde, G. Booker - personal communication). This is indicated in Figure 5.9.

Antibody 60 was shown to bind to CLPC whether avidin was complexed to the enzyme or not. In addition the presence of avidin or anti-biotin antibodies in solution does not affect the binding of this antibody to SLPC. This suggests that antibody 60 does not bind within the avidin binding site or the area covered by the binding of an anti-biotin antibody. Antibody 60 does not inhibit enzyme activity. Together, this evidence suggests that antibody 60 does not bind near the active site of the enzyme. In addition, this antibody bound equally well to TNP modified enzyme whether anti-TNP antibodies were present or not, suggesting that this antibody does not bind at the middle third of the external face of the enzyme subunit (see section 4.2.2.3). Electron micrographs of SLPC-antibody 60 complexes indicated that the antibody binds near the ends of the subunit. Since antibody 18 seemed to protrude more from the ends of the subunits in the SLPC-antibody 18 complexes than antibody 60 on SLPC, the area of binding of antibody 60 has been designated as shown in Figure 5.9.

Antibody 18 was shown to be an inhibitor of SLPC only. It was not an anti-biotin antibody and its inhibition was not affected by the presence of substrates or products (see sections 4.2.1.3 and 4.2.1.4). Anti-biotin antibodies, avidin and anti-TNP antibodies do not affect the binding of this antibody to SLPC or TNBS modified enzyme (see section 4.2.2.3). On the basis of these results it would be tempting to assign the binding of antibody 18 near antibody 6 or 113 binding sites. However electron micrographs of antibody 18-SLPC complexes reveal that these antibodies bind on the ends of the enzyme subunits. No explanation can

be offered for this apparently anomalous result, except to suggest that antibody 18 may induce a conformational change that is transmitted over the entire length of the subunit to the active site.

5.4 GENERAL DISCUSSION

In this work Fab fragments obtained from polyclonal IgG antibodies developed against the biotinyl moiety were successfully used to locate the area of the specific epitope, biotin, on the surface of the SLPC subunit. Under the electron microscope the resolving power was such that a molecule of molecular weight 25 kDa (Fab fragment) was easily observed. Anti-biotin antibodies and avidin have been used successfully to locate, by electron microscopic examination, the biotin prosthetic group on the biotin enzyme transcarboxylase. (Green et al., 1972 and Harmon et al., 1980). The anti-biotin antibodies purified by affinity chromatography on lipoyl-Sepharose were shown to bind to the small subunit of transcarboxylase. In this study the transcarboxylase-antibody complexes were extremely unstable. This was also observed for the SLPC-anti-biotin Fab complexes where often dimers, monomers and broken particles could be observed after the gel filtration step even though acetyl-CoA was present. Crosslinking of the enzyme with DTSP could overcome this problem (as was done for the monoclonal antibody study).

It would also be of interest to determine the stoichiometry of binding of the anti-biotin Fab to the enzyme, in order to confirm the proposition that only one anti-biotin Fab can bind to a dimer of SLPC in the tetramer, due to steric hindrance. To do this it would be necessary to isolate the anti-biotin specific Fab fragments from the polyclonal mixture by affinity chromatography on lipoyl-Sepharose (Harmon et al., 1980). Alternatively, monoclonal antibodies against the biotin moiety would be even more suitable.

Taking the biochemical data presented in Chapter 4 in conjunction with the electron microscopic results, a putative position of binding for each of the monoclonal antibodies was assigned. The limitation of this method was that the resolution of the electron micrographs was such that assignments could only be made to relatively large areas on the enzyme (e.g. to the central part of the subunit which is close to the inter subunit junction, to the mid-third of the subunit or to the ends or far third of the subunit). Provided that the resolution in the electron microscope is as good (as was shown for the anti-biotin Fab fragments) the use of the small Fab fragments would be expected to be more informative than using larger intact IgG molecules to probe the surface of the SLPC molecule. For example antibodies 12 and 42 are able to inhibit the activity of both chicken and sheep liver pyruvate carboxylase whereas antibodies 6 and 113 inhibit only the sheep enzyme. All of these antibodies appear to bind close to the inter subunit junction of SLPC. Electron microscopy using Fab fragments of these antibodies may distinguish more clearly between the areas of binding of these antibodies on sheep liver pyruvate carboxylase. To this end, it is the aim of the biotin enzyme research group to prepare Fab fragments of each of the monoclonal antibodies so that a more accurate binding location on the enzyme can be assigned.

A useful extension of this study would be to determine the locus of binding of each monoclonal antibody in relation to the others. Using this technique, the exclusion of binding of a second antibody to pyruvate carboxylase after a first has been bound would indicate either that the binding of the first antibody has caused some type of conformational change which has affected the binding of the second antibody, or more likely, that both antibodies have binding sites that are so close that steric hindrance prevents both from binding at one site. Assuming an area

of exclusion of approximately 3.5 nm in diameter by an antibody, (Tzartos et al., 1981) an epitope map of pyruvate carboxylase may be constructed provided that one of the epitopes has a location on the protein which is known (e.g. the biotin prosthetic group). This map could be compared with the assignments deduced from the electron microscopic and biochemical studies described in this chapter.

Figure 5.1 HPLC Gel Filtration Profiles of SLPC, anti-biotin-Fab Fragments and SLPC : Fab Mixtures

Gel filtration profiles of the following proteins or protein mixtures on a TSK G4000 SW HPLC column run in 0.1M Tris-Cl, 0.1M KCl, 100μM acetyl-CoA pH 7.2 (flow rate: 0.1ml.min^{-1}, wavelength: 215nm) are displayed:

(a) Sheep liver pyruvate carboxylase (50μg, s.a. = 30 units/mg).

(b) Anti-biotin-Fab fragments (250μg).

(c) Sheep liver pyruvate carboxylase (50μg, s.a. = 30 units/mg) incubated with 250μg of anti-biotin-Fab fragments in 0.1M Tris-Cl, 0.1M KCl, pH 7.2 at 30°C for one hour.

FIGURE 5.1

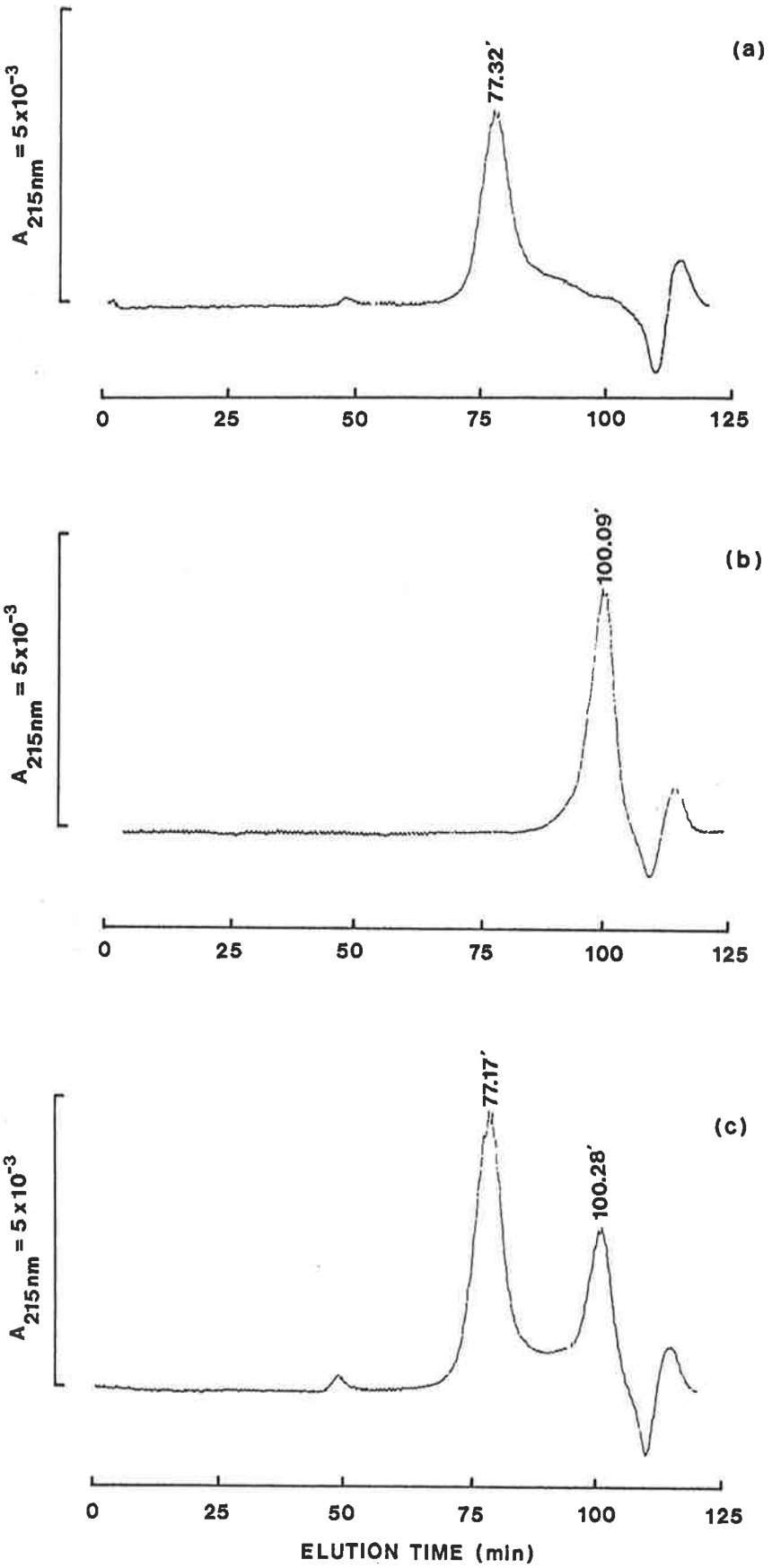

(a)
77.32'
$A_{215nm} = 5 \times 10^{-3}$
0
25
50
75
100
125
(b)
100.09'
$A_{215nm} = 5 \times 10^{-3}$
0
25
50
75
100
125
(c)
77.17'
100.28'
$A_{215nm} = 5 \times 10^{-3}$
0
25
50
75
100
125
ELUTION TIME (min)

Figure 5.2 Electron Micrographs of SLPC and Anti-biotin Fab Fragments complexed to SLPC

Figure 5.2(a) shows a field view of SLPC mounted in the presence of acetyl-CoA (250 μM). Three different projections of SLPC (Types 2, 3 and 4) are circled. The visible subunits of the tetrameric SLPC molecules are indicated by arrows. The Type 2 projections are side views of the tetramer. Two protein masses are observed representing two partially superimposed enzyme subunits. The Type 3 projections show three protein masses representing three enzyme subunits. The fourth subunit is hidden behind the three subunits pictured. The Type 4 projection shows the SLPC molecule oriented in such a way that all four subunits are visible. The bar represents 50nm.

Figure 5.2(b) shows a gallery of Type 2, 3 and 4 projections of the SLPC tetramer.

Figure 5.2(c) shows a field view of anti-biotin IgG Fab fragments (circled). The two protein masses in each Fab fragment are indicated by arrows.

In Figure 5.2(d) SLPC-Fab complexes (prepared as described in section 5.2.5) are pictured in two different orientations. The upper row shows complexes with a triangular projection of the enzyme. The lower row comprises of complexes where a 'side-on' view of the enzyme is observed. The protein masses of the enzyme are indicated by filled arrowheads. The bound Fab fragments are pictured by open arrowheads.

For Figures 5.2(b), (c) and (d) the bar represents 25nm.

Figure 5.2(e) shows a diagrammatic representation of the micrographs from Figure 5.2(d). The enzyme subunits are drawn as solid black areas while the bound Fab fragments are pictured by their contours only.

FIGURE 5.2

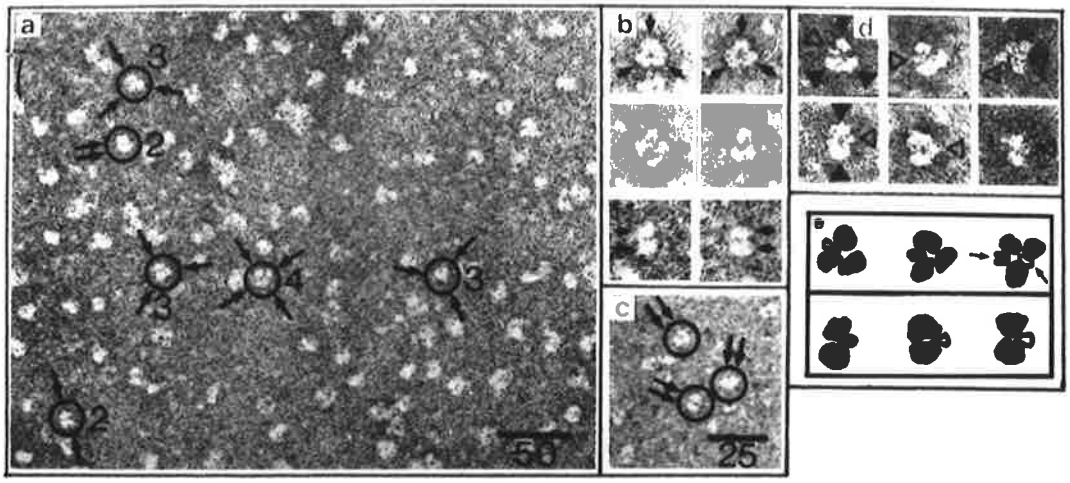


Figure 5.3 Electron Micrographs of SLPC ± Crosslinking with DTSP Diluted in the Absence of Acetyl-CoA

SLPC unmodified or crosslinked with DTSP, was diluted in the absence of acetyl-CoA and mounted for electron microscopy as described in section 5.2.6. Field views of crosslinked (a) and unmodified enzyme (b) are shown.

In Figure 5.3(a) examples of two different projections (Types 3 and 4) of SLPC which show the intact molecule are circled. The Type 3 projections show three protein masses representing three enzyme subunits. The fourth subunit is hidden behind the three visible subunits. The Type 4 projection shows SLPC oriented in such a way that all four subunits are visible.

In Figure 5.3(b) few intact tetramers are visible. The bar represents 40nm.

FIGURE 5.3

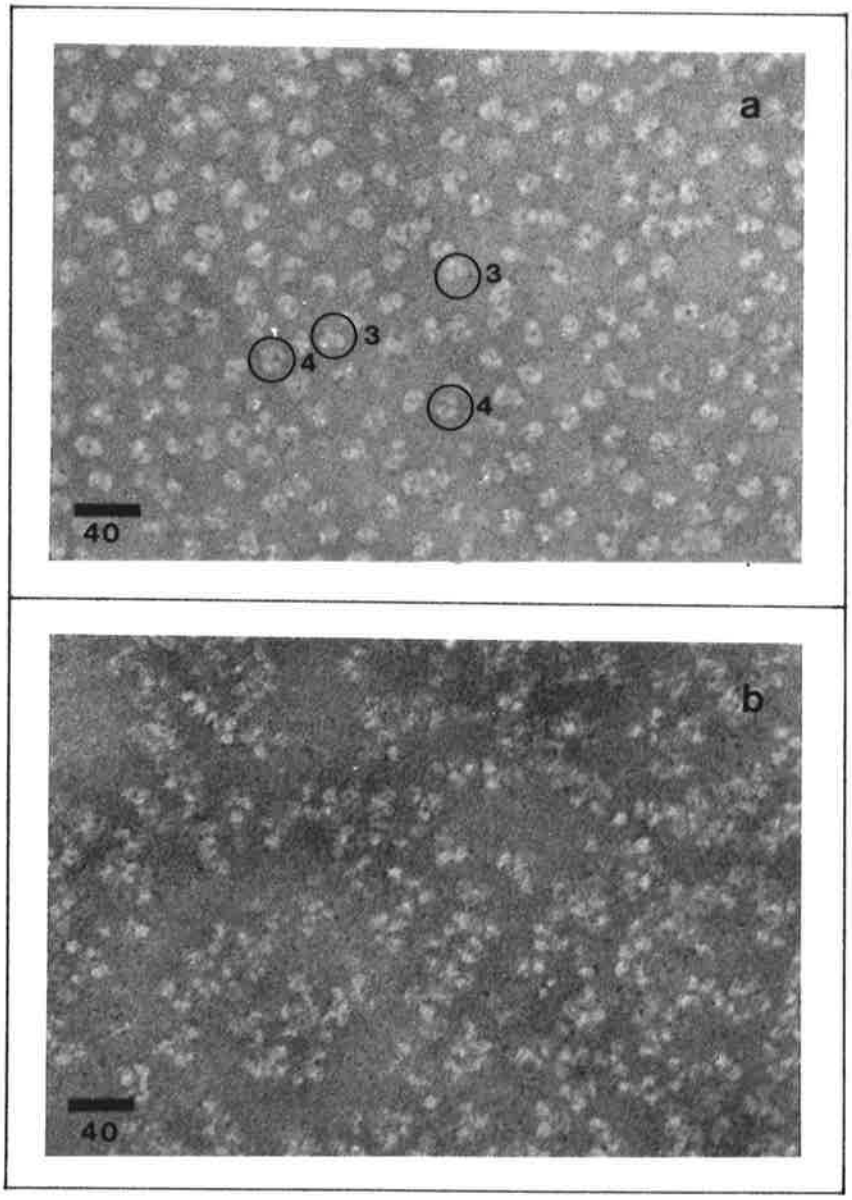
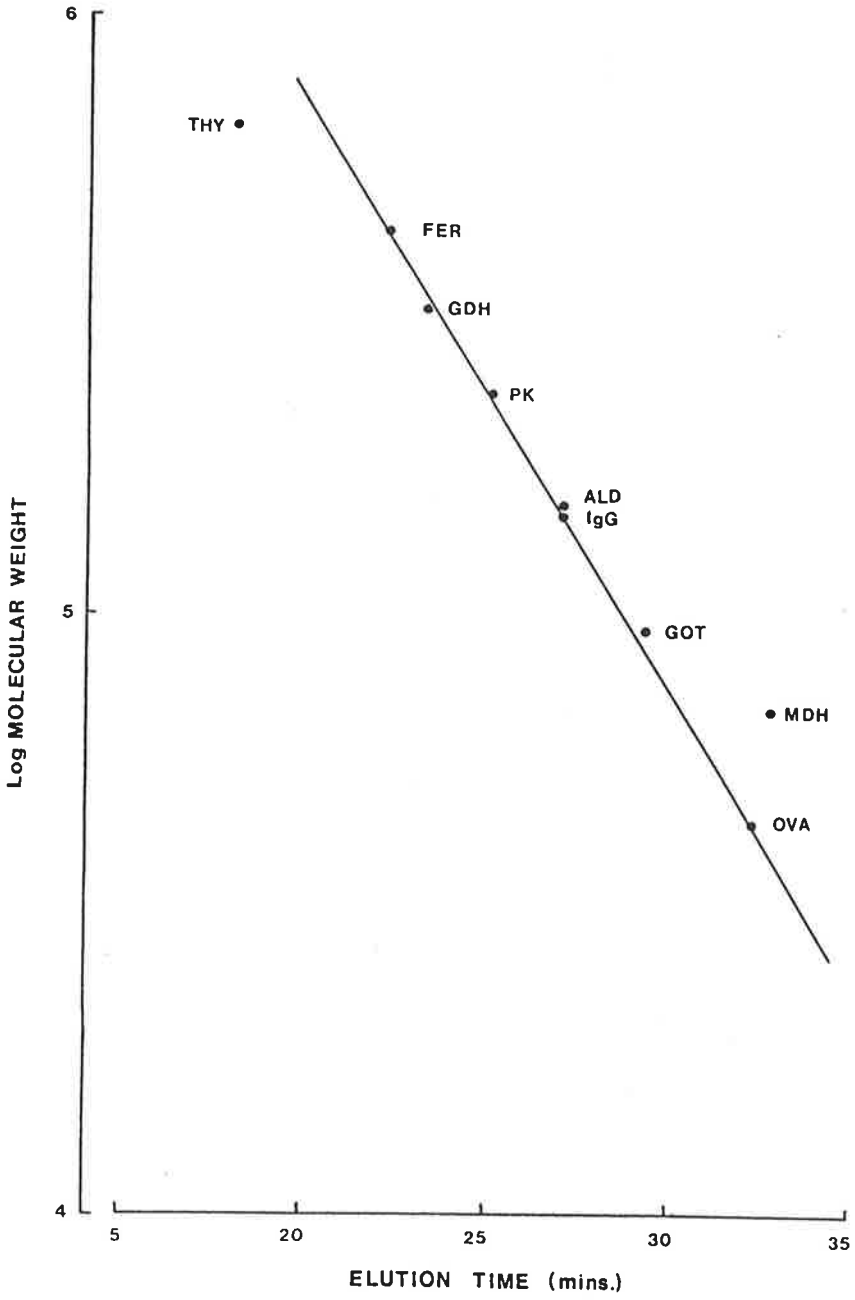


FIGURE 5.4 CALIBRATION OF TSK G3000 SW



A TSK G3000 SW gel filtration column was calibrated in 0.1M Tris-Cl, 0.1M KCl pH 7.2 at a flow rate of 0.5ml min^{-1} with the following molecular weight standards: thyroblobulin (THY) - M_r = 669,000; ferritin (FER) - M_r = 440,000; glutamate dehydrogenase (GDH) - M_r = 330,000; pyruvate kinase (PK) - M_r = 237,000; aldolase (ALD) - M_r = 158,000; IgG - M_r = 150,000; glutamate oxaloacetate transaminase (GOT) - M_r = 95,000; malic dehydrogenase (MDH) - M_r = 70,000 and ovalbumin (OVA) - M_r = 45,000. Protein eluting from the column was detected at a wavelength of 280nm.

Figure 5.5(a) HPLC Gel Filtration Profiles of SLPC and SLPC : Monoclonal Antibody (6, 12, 18) Mixtures

The following samples were resolved on an HPLC TSK G3000 SW gel filtration column run at 0.5ml min^{-1} in 0.1M Tris-Cl, 0.1M KCl pH 7.2:

(i) SLPC alone (100μg crosslinked with DTSP) in 0.1M sodium phosphate pH 7.0.

(ii) SLPC (100μg crosslinked with DTSP) incubated with monoclonal antibodies 6 (500μg), 12 (250μg) or 18 (500μg) in 0.1M sodium phosphate pH 7.0 for 30 minutes at 37°C.

Protein eluting from the column was detected at a wavelength of 280nm.

FIGURE 5.5 a

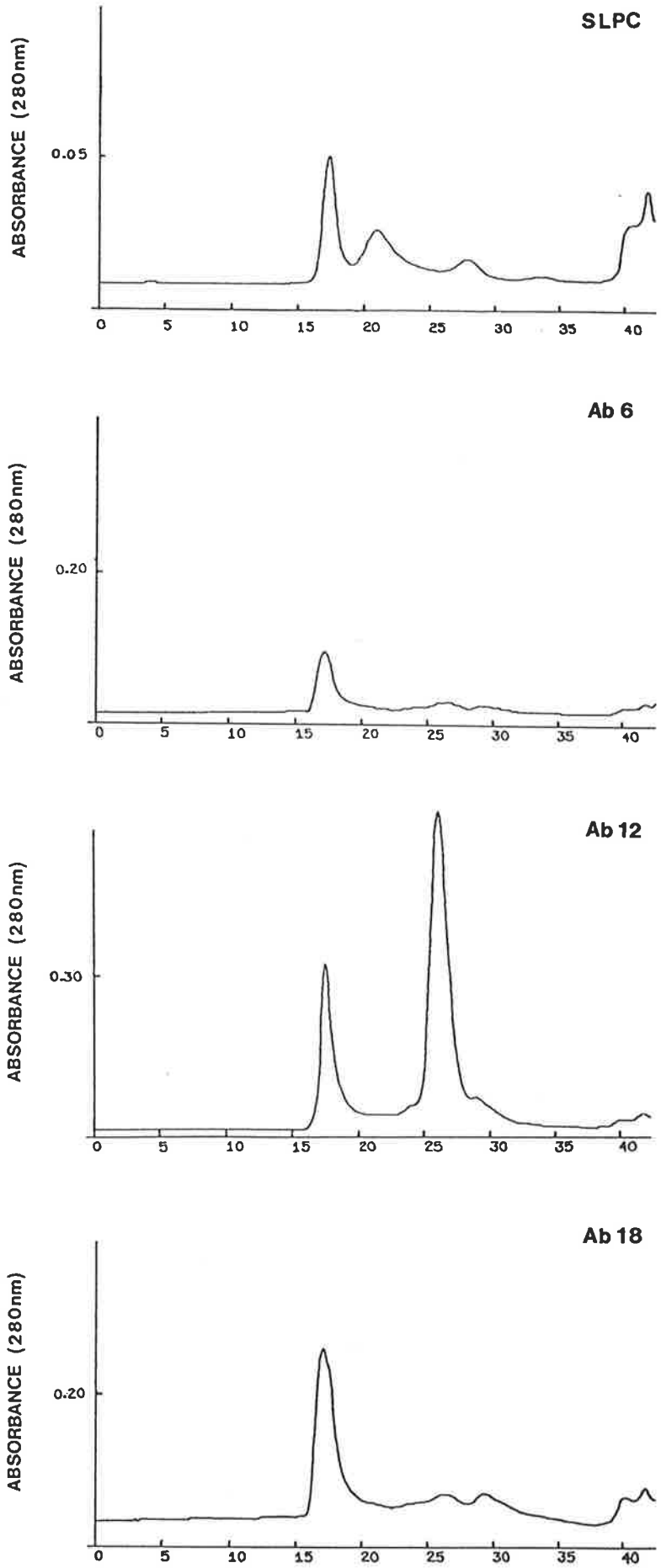

SLPC
ABSORBANCE (280nm)
0.05
0 5 10 15 20 25 30 35 40
Ab 6
ABSORBANCE (280nm)
0.20
0 5 10 15 20 25 30 35 40
Ab 12
ABSORBANCE (280nm)
0.30
0 5 10 15 20 25 30 35 40
Ab 18
ABSORBANCE (280nm)
0.20
0 5 10 15 20 25 30 35 40
ELUTION TIME (min)

Figure 5.5(b) Gel Filtration Profiles of SLPC and SLPC : Monoclonal Antibody (42, 60, 80, 113) Mixtures

The following samples were resolved on an HPLC TSK G3000 SW gel filtration column run at 0.5ml min^{-1} in 0.1M Tris-Cl, 0.1M KCl pH 7.2: SLPC (100μg crosslinked with DTSP) in 0.1M sodium phosphate pH 7.0 incubated with monoclonal antibodies 42 (200μg), 60 (500μg), 80 (500μg) or 113 (500μg) in 0.1M sodium phosphate pH 7.0 for 30 minutes at 37°C. Protein eluting from the column was detected at a wavelength of 280nm.

FIGURE 5.5 b

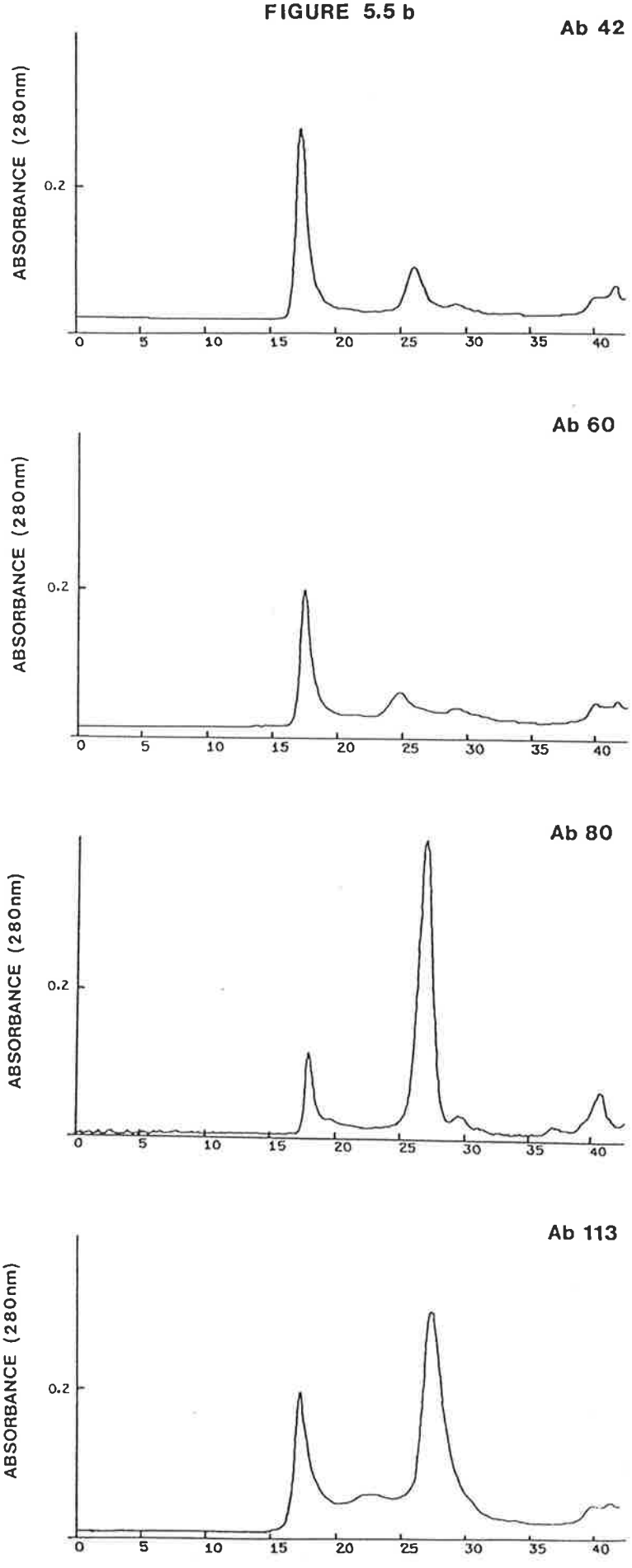
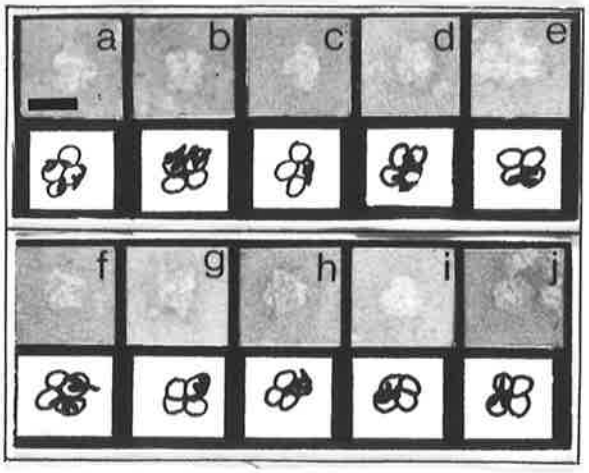

Ab 42
Ab 60
Ab 80
Ab 113
ABSORBANCE (280nm)
0.2
0
5
10
15
20
25
30
35
40
ELUTION TIME (min)

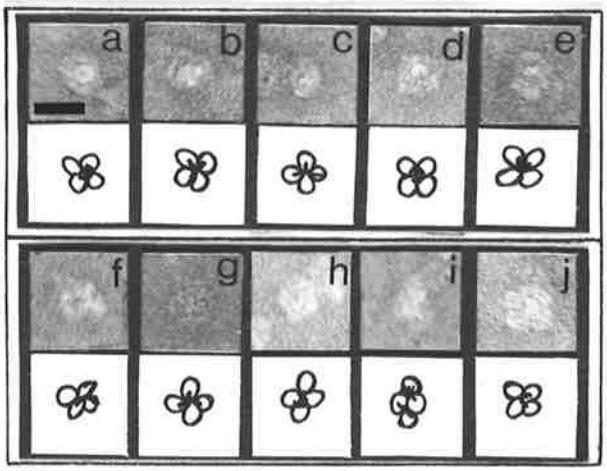
Figure 5.6 Electron Micrographs of SLPC Complexed to Monoclonal Antibodies 6, 12 and 18

Complexes of SLPC and monoclonal antibodies 6, 12 or 18 were isolated and prepared for electron microscopy as described in sections 5.2.5 and 5.2.6. Micrographs of SLPC complexed with monoclonal antibodies 6, 12 and 18 are shown. The bar represents 20nm. Beneath each micrograph the complex is represented diagrammatically. The bound antibody molecules are drawn as solid black areas. The enzyme subunits are pictured by their contours only.

Monoclonal Ab.6 + SLPC



Monoclonal Ab.12 + SLPC



Monoclonal Ab.18 + SLPC

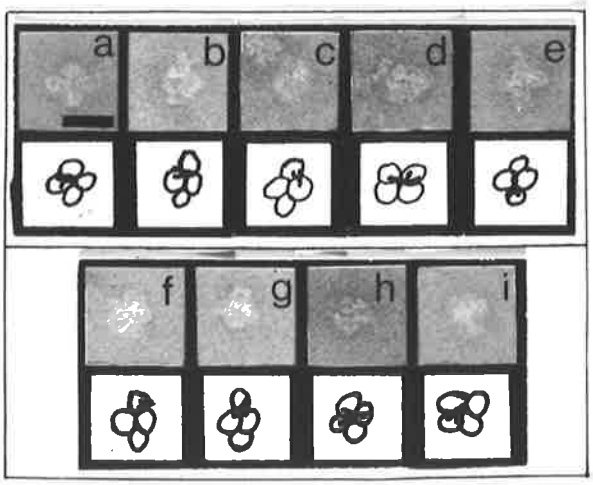
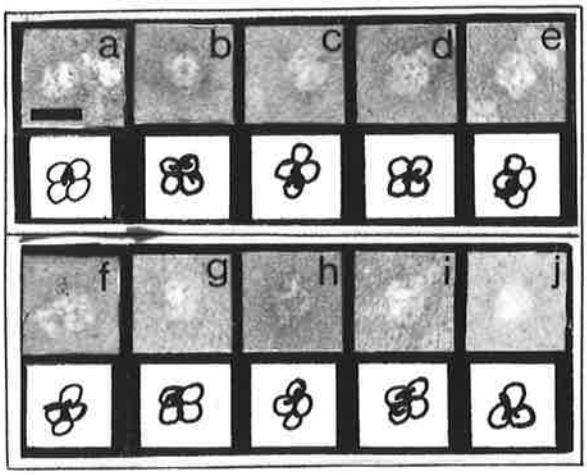


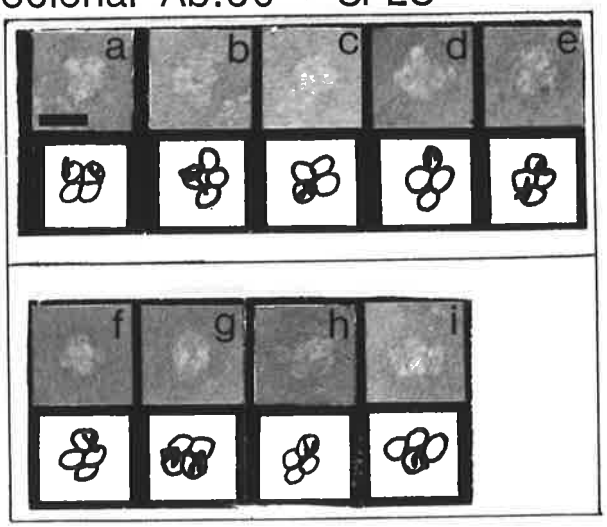
Figure 5.7 Electron Micrographs of SLPC Complexed to Monoclonal Antibodies 42, 60 and 80

Complexes of SLPC and monoclonal antibodies 42, 60 and 80 were isolated and prepared for electron microscopy as described in sections 5.2.5 and 5.2.6. Micographs of SLPC complexed with monoclonal antibodies 42, 60 and 80 are shown. The bar represents 20nm. Beneath each micrograph the complex is represented diagrammatically. The bound antibody molecules are drawn as solid black areas. The enzyme subunits are pictured by their contours only.

Monoclonal Ab.42 + SLPC



Monoclonal Ab.60 + SPLC



Monoclonal Ab.80 + SLPC

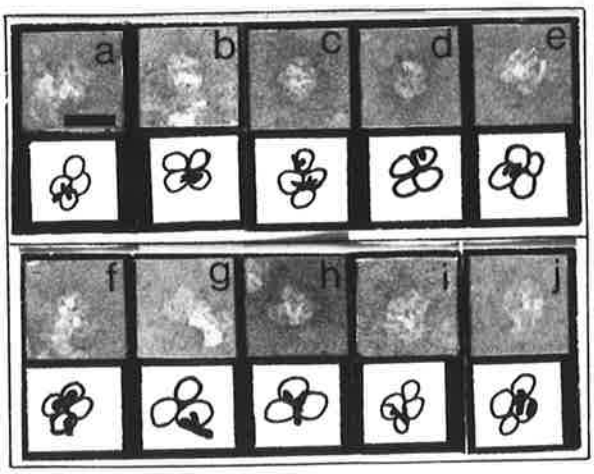


Figure 5.8 Electron Micrographs of SLPC Complexed to Monoclonal Antibody 113

Complexes of SLPC and monoclonal antibody 113 were isolated and prepared for electron microscopy as described in sections 5.2.5 and 5.2.6. Micrographs of SLPC complexed with antibody 113 are shown. The bar represents 20nm. Beneath each micrograph the complex is represneted diagrammatically. The bound antibody molecules are drawn as solid black areas. The enzyme subunits are pictured by their contours only.

Monoclonal Ab.113 + SLPC

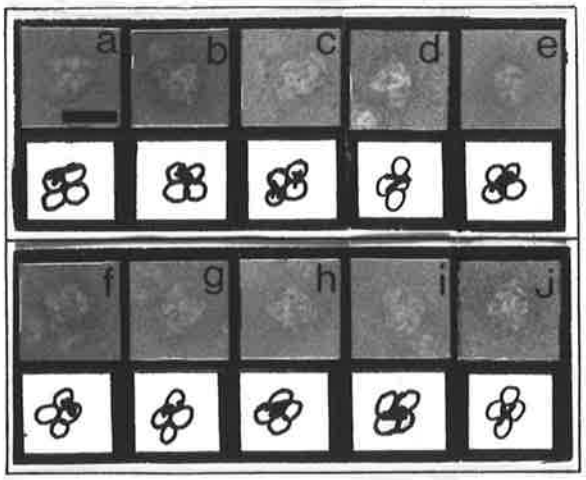
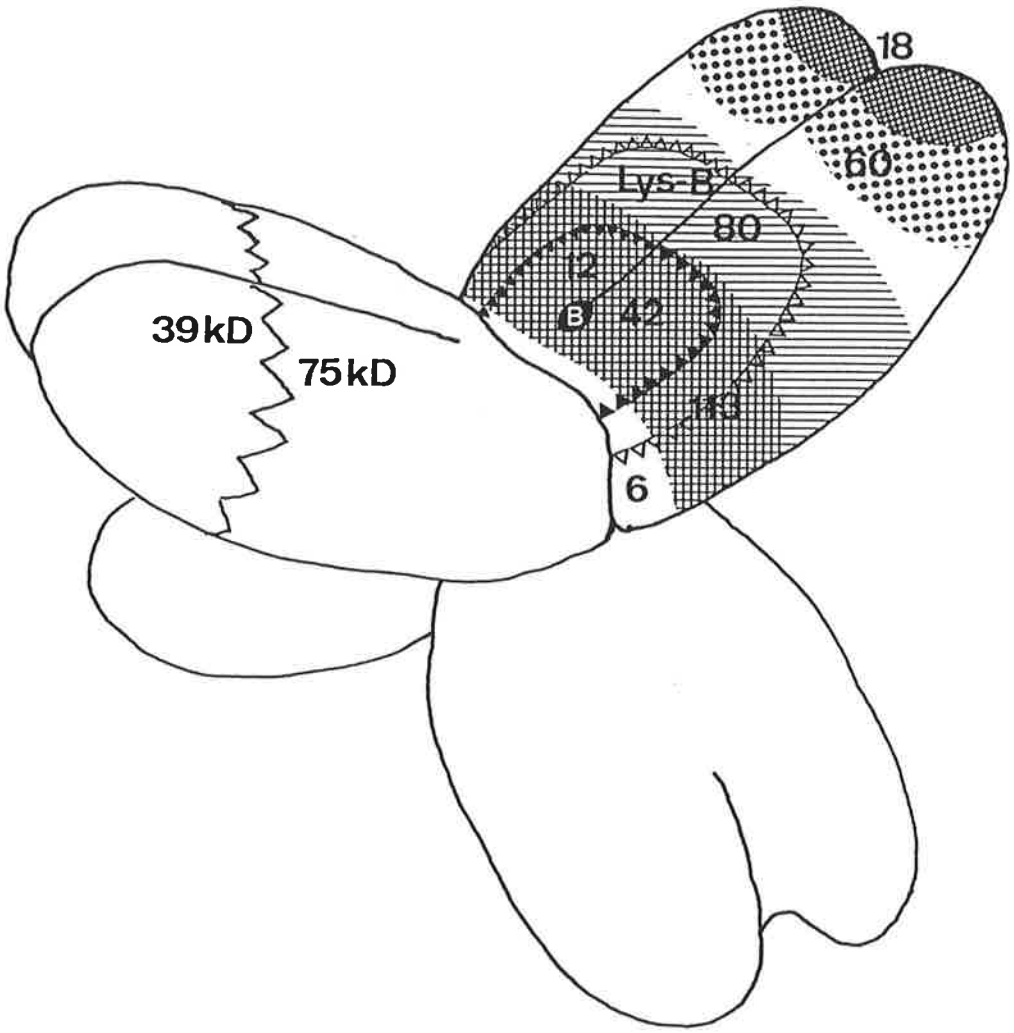


Figure 5.9 Diagram of Binding Loci of Monoclonal Antibodies on SLPC



This diagram summarises the putative binding loci of the monoclonal antibodies 6, 12, 18, 42, 60, 80 and 113 on one subunit of SLPC in relation to the biotin prosthetic group (B) and the regions occupied by anti-biotin IgG (ΔΔΔ) and avidin (▼▼▼). The position of the lysine on SLPC which is labelled by TNBS in the presence or absence of acetyl-CoA (Lys-B) is also indicated.

On the other subunit of the same dimer a chymotryptic cleavage point is shown. The significance of this is discussed in section 6.3.3.

CHAPTER 6

GENERAL DISCUSSION

GENERAL DISCUSSION

Since the results from chapters 3, 4 and 5 have been discussed previously, this chapter will serve as a brief overview of the project as a whole and the direction the work may take in the future.

6.1 PRODUCTION OF MONOCLONAL ANTIBODIES AGAINST SLPC

One of the aims of the project was to produce a panel of monoclonal antibodies against pyruvate carboxylase from sheep liver. Binding of the antibodies to the enzyme was established by ELISA methods where native enzyme was bound to microtiter plates. (It was recognised, however, that the binding of the enzyme to the plate may distort it somewhat and thus the bound enzyme may not be truly native.) Confirmation of binding to native enzyme may be obtained by incubation of active enzyme with antibody in solution followed by precipitation of the complex by anti-mouse antibody or Protein A and assaying for enzyme activity in the supernatant. Alternatively, a competition ELISA system may be employed where 'native' enzyme bound to a microtiter plate competes with enzyme in solution for the antibody which is also in solution. Nevertheless, some antibodies showed inhibition of enzyme activity and all antibodies tested were seen bound to the enzyme under the electron microscope after a period of incubation of both enzyme and antibody in solution. Some antibodies showed binding to SLPC which had been denatured and reduced by SDS and 2-mercaptoethanol prior to binding to a microtiter plate.

None of the antibodies within the first set of monoclonal antibodies produced, were able to inhibit the activity of SLPC. It was considered desirable to have antibodies which inhibited the activity of the enzyme, hence a further set of antibodies was produced where secondary screening of all the culture supernatants which bound to SLPC in ELISA, included an

assay for inhibition of enzyme activity. Indeed, many culture supernatants were found which inhibited SLPC activity. Nine subclones, five of which produced antibodies that inhibited SLPC activity were eventually characterised further.

6.2 CHARACTERISATION OF MONOCLONAL ANTIBODIES BY PHYSICAL AND BIOCHEMICAL MEANS

The characterisation of the antibodies was directed towards the localisation of the antibody binding sites on the enzyme subunit. This was achieved by determination of the position of binding of the antibodies with respect to markers of the enzyme's active site (avidin and anti-biotin antibodies); their effect on enzyme activity in the presence and absence of biotin, the substrates Mg^{2+}, $MgATP^{2-}$ and pyruvate and the product oxaloacetate; their effect on the acetyl-CoA independent activity of SLPC and their physical location by electron microscopy. Based on this investigation, an epitope map of SLPC was elucidated by the members of the biotin enzyme research group involved in this work (see Figure 5.9).

6.3. FUTURE STUDIES

6.3.1 Isotope Exchange Reactions and Electron Microscopy

To extend this investigation, isotopic exchange reactions (i.e. ATP:P_i exchange and pyruvate: oxaloacetate exchange for the first and second partial reactions respectively) may be more definitive in elucidating the part of the active site which is affected by the binding of the inhibitory antibodies. For the electron microscopy study, it is hoped that in positioning the antibody binding site on the enzyme, a

finer distinction may be gained in using Fab fragments of the antibodies. This approach is feasible since the position of binding of Fab fragments of anti-biotin antibodies to SLPC has been determined (see section 5.3.2).

6.3.2 Epitope Mapping of Monoclonal Antibodies

As discussed in chapter 5, mapping the location of the antibodies with respect to one another would be useful. This may be carried out in several ways. For example, saturating levels of antibody A are used to probe antigen bound to the wells of a microtiter plate. After addition of labelled antibody B, the amount of this antibody may then be determined. The label on antibody B may be for example, radioactivity, enzyme or biotin. For the latter two cases addition of enzyme substrate or avidin labelled with enzyme and then substrate is required to quantitate the amount of antibody B present. Alternatively if the two antibodies differ in isotype then an anti-isotype serum which will only detect antibody B can be used. If the antibodies share the same binding site or bind in close proximity to each other, there is a loss of signal from antibody B in comparison to the signal when no antibody A is present. In an alternate system, antibody A attached to a microtiter plate may be used to capture radiolabelled antigen which has been pre-incubated with a large excess of antibody B. If the antibodies share the same binding site then no radiolabel will be bound to the microtiter wells. Another procedure is to use antibody A attached to microtiter wells to capture unlabelled antigen. Subsequent binding of labelled antibody B is then determined. This approach however may not be definitive for the tetrameric SLPC molecule which would probably contain more than one epitope for each enzyme tetramer. If the capture antibody does not bind

all epitopes on the tetramer (and spatial consideration of the enzyme's structure suggest it could not in most cases), then a second antibody which shares the same antigenic determinant may still bind.

6.3.3 Limited Proteolysis Studies

Another useful technique in mapping the binding of these antibodies on pyruvate carboxylase is limited proteolysis of the enzyme, SDS-PAGE of the proteolytic cleavage followed by electroblotting and immunoprobing. This approach relies on the ability of the monoclonal antibody to recognise peptides which have been denatured and reduced and has been used for example in part of the study on rat brain hexokinase (Wilson and Smith, 1985). Monoclonal antibodies to this enzyme were used to probe a limited tryptic cleavage of rat brain hexokinase which had been separated by SDS-PAGE. Knowing the effect that these antibodies had on the various functions of the enzyme such as catalysis and membrane binding, these workers were able to use the results of the peptide mapping to build a three dimensional model of the enzyme. This model relates the structural to functional aspects of rat brain hexokinase. Alternatively iodinated enzyme may be subject to limited proteolysis followed by immunoprecipitation and SDS-PAGE. For example, radiolabelled acetylcholine receptor subunits were subject to S.aureus V8 protease digestion followed by immunoprecipitation and SDS-PAGE in order to determine the peptide to which each monoclonal antibody binds (Gullick et al., 1981).

Using SDS-PAGE, Khew-Goodall (1985) showed that digestion of sheep or chicken liver pyruvate carboxylase with chymotrypsin, trypsin or S.aureus V8 protease resulted in a major peptide of molecular mass between 75 and 83 kDa which was relatively stable to further proteolysis. For CLPC, another smaller peptide of 39 kDa was also observed. The sum of the two

peptides approximates the molecular mass of the intact subunit. On the basis of this evidence, it was suggested that the subunits of pyruvate carboxylase may consist of two major domains, one approximately two thirds of the subunit, connected by a hinge region which is susceptible to proteolytic attack. Khew-Goodall found that cleavage of the sheep and chicken enzyme in this manner, resulted in the loss of acetyl-CoA dependent overall activity. The large domain however, was found to contain all the elements of the active site including the biotin prosthetic group and all or part of the allosteric site for acetyl-CoA. Unlike the intact enzyme tetramer, it was shown that acetyl-CoA could not prevent dissociation of tetramers of 75 kDa fragments suggesting that acetyl-CoA cannot bind or if it can, it cannot exert its effects. Hence it was proposed that although the large domain was believed to contain the binding site for acetyl-CoA, the small domain was crucial for maintaining the allosteric site in the correct functional conformation. In 1983 Johannssen et al. reported that the active site of pyruvate carboxylase was close to the intersubunit junction on the intact enzyme. Therefore it follows that if the 75 kDa fragment contains the components of the active site then it must also contain the part of the subunit which is close to the intersubunit junction. Khew-Goodall suggested then that the small peptide which is removed from the subunit by limited proteolysis to yield the 75-83 kDa peptide, is distal from the intersubunit junction. Taking this evidence into account, as well as the appearance of tetramers of 75 kDa fragments under the electron microscope (Khew-Goodall - personal communication), the possible hinge site on the intact enzyme that is susceptible to proteolysis is shown in Figure 5.9.

In view of this information it would be of interest to determine which chymotryptic/tryptic/S.aureus V8 protease generated fragment each monoclonal antibody binds. This would give some support as to the placement of the antibody sites on the enzyme subunit as displayed in

Figure 5.9. Following, this further proteolysis of the fragments under denaturing conditions into peptides small enough to be sequenced and the determination of the peptides containing the antibody combining sites would provide invaluable information for this work. For example, the binding sites of those antibodies which affect the activity of the enzyme would be of special interest. These include inhibitor antibodies which bind within and outside the avidin exclusion area. Of particular interest would be antibody 18, an inhibitor of SLPC activity which appears to bind at the ends of SLPC subunits, distal from the intersubunit junction.

6.3.4 Screening of Recombinant Fusion Proteins Using Monoclonal Antibodies

In 1984, Nunberg et al. described the expression of DNA fragments of the gene encoding the feline leukemia virus envelope protein, gp70 as β-galactosidase fusion proteins. Monoclonal antibodies were used to screen the recombinant phage libraries expressing these fusion proteins. The use of antibody probes in this manner has also been carried out for example by Erlich et al., 1978, Young and Davis, 1983 and Ruther et al., 1982. Upon isolation of the complete gene for pyruvate carboxylase this type of experiment may be performed with the monoclonal antibodies available. It must be borne in mind, however, that not all antibodies which recognise the native enzyme will recognise recombinant peptides of the enzyme since the latter may not have folded into the conformation they have in the entire native protein.

Hence, information regarding the peptides to which each antibody binds, either through peptide mapping techniques or by the recombinant DNA method described, may be accumulated. In conjunction with the

biochemical and electron microscopic information presented in this thesis, a detailed epitope map of pyruvate carboxylase may be compiled relating the structural to the functional aspects of the molecule.

6.4 IMMUNOLOGICAL CROSS REACTION AMONGST BIOTIN ENZYMES

Another aspect of the Ph.D. project was to use monoclonal antibodies to study immunological cross reaction amongst the biotin enzymes. A study was carried out on antibodies produced by hybridoma cells both before and after cloning out, using an ELISA based system where the biotin enzyme was bound to the microtiter plate. The study before cloning out looked at the cross reaction of antibodies raised against SLPC amongst pyruvate carboxylase from chicken, pheasant, kangaroo and rat liver and sheep liver propionyl-CoA carboxylase. There was considerable cross reaction with all of the biotin enzymes tested with the rat enzyme being the most antigenically similar to the sheep enzyme. SLPCC was the least similar with 24% of the cell culture supernatants binding to it. The study with the monoclonal antibodies involved pyruvate carboxylase from sheep, chicken and yeast, transcarboxylase from P. shermanii and sheep liver propionyl-CoA carboxylase. Cross reaction was observed both across species for pyruvate carboxylase and to a lesser extent amongst the different biotin enzymes. The cross reaction with transcarboxylase should be pursued to see if the 5S and 1.3S subunits react since they are the more homologous regions.

Two inhibitor monoclonal antibodies (12, 42) which have been shown to bind close to, or at the active site of SLPC also inhibited CLPC activity. It would be of great interest to determine the effects that these antibodies have on the activity of other biotin carboxylase enzymes. In addition, comparison of the amino acid sequences of the peptides to which each monoclonal antibody binds for each biotin enzyme

may give some clues as to the extent of similarity of their primary structure (at least at the antigenic determinants) and the importance of particular structural features for the similar functions of the biotin enzymes.

Khew-Goodall (1985) has suggested that since the large chymotryptic fragment of chicken and sheep liver pyruvate carboxylase, the biotin containing subunits of the eukaryotic propionyl-CoA carboxylases, bovine kidney and P. citronellolis methyl-crotonyl-CoA carboxylase and geranyl-CoA carboxylase from P. citronellolis are of comparable molecular masses, i.e. 75-82 kDa (Lau et al., 1979; Goodall, 1981; Kalousek et al., 1980; Fall and Hector, 1977) that they may have common ancestral roots. To pursue this idea, a more extensive immunological study using the biotin containing fragments of these enzymes and the large chymotryptic fragment of sheep and chicken liver pyruvate carboxylase with the monoclonal antibodies described in this thesis, could be initiated.

Using polyclonal antibodies raised against denatured SLPC and SLPCC, Mottershead et al. (1984) demonstrated immunological cross reaction with several different biotin enzymes. Hence, our research group has used two different approaches to demonstrate that there are at least some antigenic determinants which are shared amongst the biotin enzymes. Ultimately, DNA sequencing of the genes for the biotin enzymes and their determination of crystalline structures will give greater insight as to the similarity of the primary and quarternary structure of the different biotin enzymes and perhaps some clues as to their evolutionary roots.

APPENDICES

APPENDIX A

HAT selection for hybrid cells

The fusion of NS-1 cells with immune spleen cells results in a mixture of hybrid cells, mainly NS-1:NS1, NS1:spleen and spleen:spleen cells. Because the incidence of a NS-1:spleen cell hybrid is very small (approximately 1 in 2 x 10^5 spleen cells), these hybrids would almost certainly die due to competition with myeloma cells. Hence it is required to kill the NS-1 cells and NS-1:NS-1 hybrids but leave the NS-1:spleen cell hybrids intact. This is achieved by growing the fusions in hypoxanthine, aminopterin and thymidine medium (HAT) which selectively kills the NS-1 cells or NS-1:NS-1 hybrids. The remaining unfused spleen cells die fairly rapidly as they require sophisticated culture conditions for survival.

The HAT selection works as follows:
The aminopterin in the HAT medium blocks the de novo synthesis of purine and pyrimidine nucleotides in the cell by acting as a substrate analogue for the enzyme dihydrofolate reductase which is necessary for the eventual generation of N^5, N^{10} methylene tetrahydrofolate from dihydrofolate. This is an essential step in the synthesis of all nucleotides. However, while the aminopterin prevents nucleotide biosynthesis by the standard cell pathway, the cell can manufacture its purine nucleotides via a 'salvage pathway' by reprocessing old nucleotides. The enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT) is a key enzyme in this pathway which uses the exogenous

hypoxanthine. The NS-1 cells lack HGPRT, hence NS-1 and NS-1:NS-1 fused cells cannot grow when the de novo nucleotide synthesis is blocked by aminopterin in the HAT Medium. The only cells which contain HGPRT are the spleen cells and the NS-1:spleen cell hybrids. The NS-1:spleen cell hybrids obtain HGPRT from the spleen cell which allows the hybrid to utilise the exogenous hypoxanthine (via the 'salvage pathway') and the thymidine (which is a source of pyrimidine for nucleotide synthesis) and thus grow in HAT medium.

APPENDIX B

Parental Myeloma Cell Line: NS-1

The cell line P3-NS-1-1-Ag4 (abbreviated NS-1) is derived from MOPC-21, a BALB/c myeloma cell line. It is 8-azaguanine resistant and susceptible to HAT Medium (see Appendix A). NS-1 cells do not produce the MOPC-21 γ_1, heavy chain. The MOPC-21 κ chain is synthesised but not secreted. However, antibody producing hybrid cell lines derived from NS-1 may produce antibodies which contain the κ light chain. By using the NS-1 cell line for fusion, three mixtures of immunoglobulin heavy and light chains are possible. All contain the heavy chains (H) of the spleen cell immunoglobulin and either a mixture of the κ chain of the NS-1 and the light chain (L) of spleen cell immunoglobulin, two NS-1 κ chains or two spleen cell light chains eg H_2LK, H_2K_2 or H_2L_2. Antibody activity is limited to the two species which contain the spleen cell light chain.

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